THE ROLE OF EARTHWORM GUT-ASSOCIATED MICROORGANISMS IN THE FATE OF PRIONS IN SOIL

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Summary

The earthworm-associated microbial communities were studied for their ability to degrade a recombinant PrP used as a model of the agent of transmissible spongiform encephalopathy (TSE).
Initially the microbial compositions of substrata (soddy-podzolic soil and horse manure compost) and their changes upon the passage through the guts of earthworms (species *Lumbricus terrestris*, *Aporrectodea caliginosa*, and *Eisenia fetida*) and the bacterial composition of the earthworm gut environment were studied using rRNA-based techniques, fluorescence *in situ* hybridization (FISH) and PCR-based approaches (cloning and single strand conformation polymorphism (SSCP) analyses).
In the most cases the number of physiologically active bacteria, i.e. those hybridized with universal FISH probe, was slightly higher in the earthworm casts in comparison to substrata. Bacterial populations of substrata were undergoing severe alterations upon transit through the earthworm gut depending mainly of the initial microbial composition presented in the substrata, in contrast to that, earthworm species-specific effects on the bacterial population composition were not detected. Certain common regularities of microbial population modification upon passage were noticed.
Clone libraries of substrata (soddy-podzolic soil and compost) and earthworm-derived systems (gut and cast) revealed a high diversity of microorganisms. Phylum *Proteobacteria* was the most diverse among the others; *CFB* was the second numerous taxon. Except of the bacteria, the eukarya (fungi (*Ascomycota*), algae (*Chlorophyta*), *Colpodae* (*Protozoa*), *Monocystidae* (*Alveolata*), and roundworm (*Nematoda*)) were detected in the substrata and earthworm-derived systems.
Application the SSCP analysis with universal and newly designed taxon-specific primers targeting α-, β- and γ-*Proteobacteria*, *Myxococccales* (δ-*Proteobacteria*), *CFB* group, *Bacilli*, *Verrucomicrobia*, and *Planctomycetes* identified the bacterial groups sensitive to, resistant against, and promoted by earthworm gut environment whereas significant differences between rRNA gene and rRNA pools were observed for all bacterial taxa except of *CFB* bacterial group.
Novel family '*Lumbricoplasmataceae'* within the class *Mollicutes* (*Firmicutes*) was proposed after detection in the gut and cast clone libraries of a monophyletic cluster of sequences and *in situ* detection with specific oligonucleotide probe by FISH analysis in the earthworm tissues.
Our data suggests the bacteria from the group *CFB* are promoted by the gut environment, the bacteria of family '*Lumbricoplasmataceae'* are obligate earthworm-associated organisms, for *Gammaproteobacteria* and *Bacilli* gut environment is hostile, although bacteria of the genus *Pseudomonas*, family of unclassified *Sphingomonadaceae* (*Alphaproteobacteria*), and

Alcaligenes faecalis (*Betaproteobacteria*) could be designated as gut-resistant component of community.

Pure microbial cultures and water-soluble content from the soil and earthworm casts of *L. terrestris* and *A. caliginosa* were elucidated for their abilities to digest recombinant prion. Up to 20% of bacterial species were able to digest recPrP; *Gammaproteobacteria*, *Actinobacteria*, and *Bacilli* were the taxa with the biggest potential to deplete the recPrP. Most of studied fungal isolates did perform the recPrP digestion.

RecPrP was demonstrated to be depleted *in vitro* in aqueous extracts of the soil and the cast within 2-6 days. Non-specific proteolytic activity strongly increased from soil substratum to the cast through the trypsin- and chymotrypsin-like proteases released by the earthworm. However, the passage through the gut did not promote any enhanced recPrP digestion, which lasted under given conditions *in vitro* within 2-6 days. Thus, under applied conditions the microbial-earthworm gut systems do not produce proteases *de novo*, which notably affect the prion proteolysis.

1. Introduction

Prion diseases: early hypotheses

The group of prion diseases also known as transmissible spongiform encephalopathy (TSE) includes the most well-known human prion diseases (kuru, Creutzfeldt-Jakob disease (CJD), and its variants (familial, sporadic, latrogenic, fatal familial insomnia and the new variant of CJD (vCJD) (Will *et al.* (1996)) and bovine spongiform encephalopathy (BSE) of cattle and scrapie of sheep and goats. Besides, this kind of prion disease was recognized in deer and elk species in North America and named chronic wasting disease (CWD); in greater kudu (Kirkwood *et al.,* 1993); in zoological ruminants and non-human primates (Bons *et al.,* 1999); feline spongiform encephalopathy of zoological and domestic cats (FSE) (Pearson *et al.,* 1992), and in other predator mammalians: transmissible mink encephalopathy (TME) (Marsh and Hadlow, 1992). On the basis of following analyses it was suggested that these apparently novel TSEs including vCJD had the same origin – BSE (Collinge *et al.,* 1996; Collinge, 1999). All of these variants of the prion diseases cause a progressive degeneration of the central nerve system ending in inevitable death.

The transmissibility of the TSEs was accidentally demonstrated in 1937, when the population of Scottish sheep was inoculated against the common virus with extract of the brain tissue unknowingly derived from a scrapie animal. In humans, kuru was emerged at the beginning of the 1900s among the cannibalistic tribes of New Guinea, reached epidemic proportions in the mid-1950s and disappeared progressively in the latter half of the century to complete absence at the end of the 1990s. The transmissibility of kuru to monkeys was demonstrated in the 1960s (Gajdusek *et al.,* 1966, Gibbs *et al.,* 1968). The CWD was first noticed at the late 1960s in Colorado wildlife research facility and later was identified as spongiform encefalopathy-forming disease according to the histological studies (William and Yang, 1980).

The exact nature of the transmissible pathogen has been debated since the mid-1960s. The incubation period of prion diseases is unusually long (up to several years) and the agent was initially thought to be a slow virus (Cho, 1976). Further research, however, has emphasized that the agent responsible for scrapie was very resistant to UV and ionizing radiation,i.e. against the treatments that normally destroy nucleic acids (Alper *et al.,* 1967). The other hypothesis, so called "virino hypothesis", suggested the presence of an agent-specific nucleic acid enveloped in a host-specified protein (Fig. 1A-c). This concept was proposed to explain the lack of an immune response by the host along with the strain variation (Kimberlin, 1982). The virus and virino hypotheses have apparently lost their importance (though have not been neglected completely) since many studies

conducted in numerouslaboratories could not identify either TSE-specific or TSE-associated nucleic acids, or nucleic acid that could encode a small protein (Riesner *et al.*, 1993).

The basics of the prion concept

A dominating current hypothesis is the 'protein-only' (or 'prion') hypothesis (Griffith, 1967), whereas a hypothetical protein is believed to comprise the entire infective particle (Fig. 1A-b). Soon after publication of the prion hypothesis, the PrP protein was discovered (Bolton *et al.,* 1982). Another experimental evidence of the hypothesis was the demonstration that PrP and TSE infectivity were co-purified (Diringer *et al.* 1983) and it was suggested that TSE infections are caused by an infectious protein, PrP (McKinley *et al.,* 1983).

The DNA encoding PrP was detected, sequenced and the prion was recognized as a host glycoprotein with unknown function (Oesch *et al.* 1985). The primary structure of the mature PrP protein comprises approximately 210 amino acids. It has two N-glycosylation sites (Oesch *et al.,* 1985) and a C-terminal glycophosphoinositol (GPI) anchor (Stahl *et al.,* 1990b). PrP mRNA proved to be the product of a single host gene, which is present in the brain of uninfected animals and is constitutively expressed by many cell types. One distinguishes two PrP forms according to the their biochemical properties. The physiologically occurring PrP^{C} fraction attached with GPI to the outer surface of the plasma membrane (Fig. 1A-a); the PrP^{C} can be glycosylated on one or both of two asparagine residues with a variety of glycans; soluble in detergents (Meyer *et al.,* 1986) and released from the surface of tissue culture cells by phosphoinositol phospholipase C (PIPLC) (Stahl *et al.,* 1990a). PrP^{C} is suggested to be the normal form of the protein, present in both uninfected and infected tissues. An isoform named PrP^{Sc} almost invariably detected in TSE-infected tissues and cells is not released by PIPLC (Stahl *et al.,* 1990a). Both normal and scrapie isoforms of PrP encoded by the same gene *Prnp* (Basler *et al.,* 1986) and it has been proposed that the normal prion PrP^{C} converses itself into 'multipling' infectious agent PrP^{Sc} (Prusiner, 1991).

There is however no evidence of structural differences between the normal PrP^{C} and isoform PrP^{Sc} (Stahl *et al.,* 1993). The normal PrP^{C} has three α-helices and one small region of β-sheet, while abnormal isoform PrP^{Sc} has a higher degree of β-sheet (Riek *et al.* 1996). These structural changes cause the alterations in biochemical properties, such as protease resistance, and capability to form larger-order aggregates. The PrP^{C} fraction is protease-sensitive. The PrP^{Sc} fraction detected only in TSE-affected tissues is partially protease-resistant (Meyer *et al.,* 1986).

It should be noted that 'protease resistance' is rather relative definition: some forms of PrP are more resistant to treatment with proteinase K (PK) than PrP^{C} but are nonetheless non-infectious (Post *et al.* 1998; Appel et al., 1999).

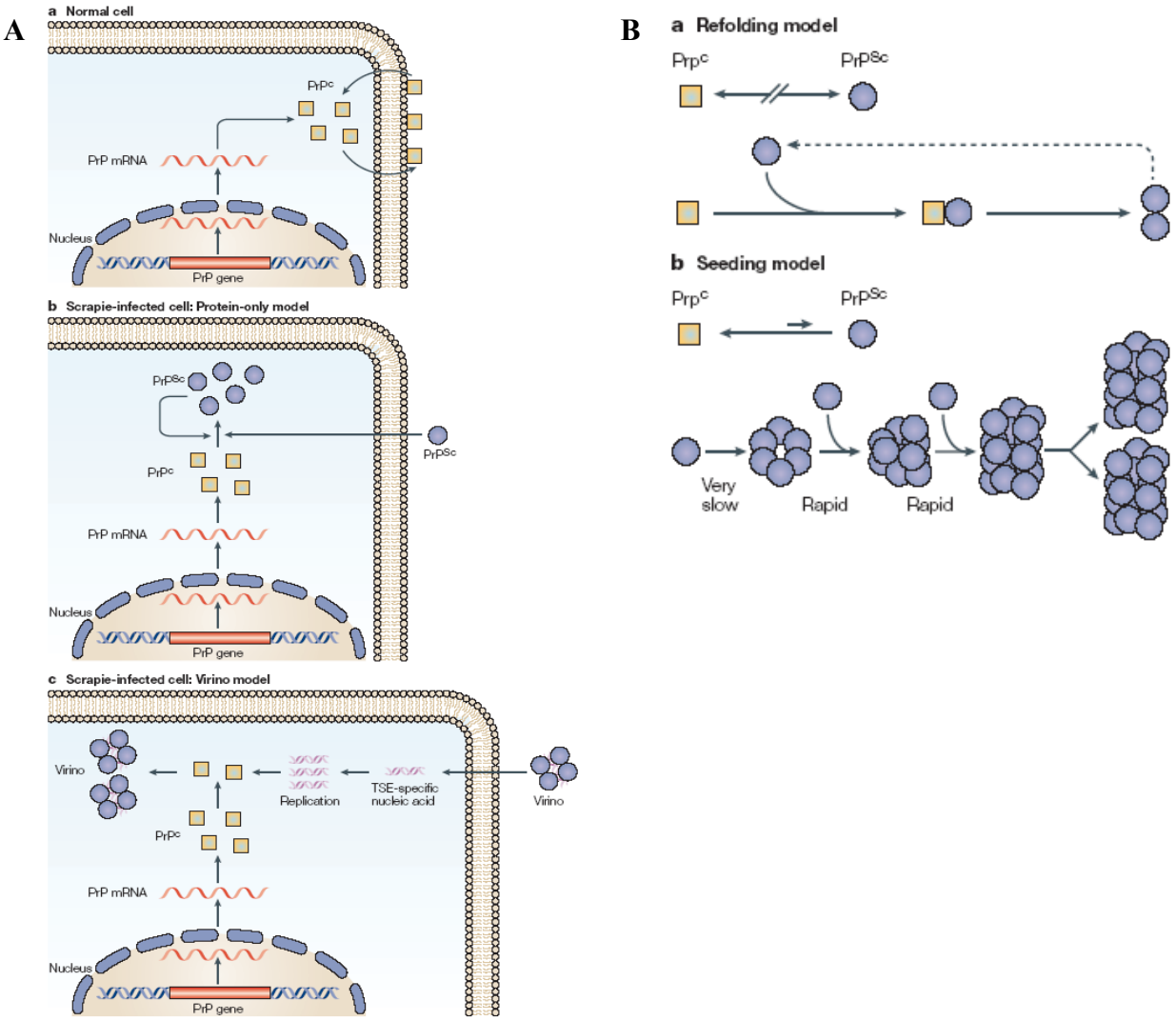


Figure 1. A: Models for the propagation of the TSE agent (prion). **a)** In a normal cell, PrP^{C} (yellow square) is synthesized, transported to the cell surface and eventually internalized. **b)** The protein-only model postulates that the infectious entity, the prion, is congruent with an isoform of PrP, here designated as PrP^{Sc} (blue circle). Exogenous PrP^{Sc} causes catalytic conversion of PrP^{C} to PrP^{Sc}, either at the cell surface or after internalization. **c)**The virino model postulates that the infectious agent consists of a TSE-specific nucleic acid associated with or packaged in PrP^{Sc}. The hypothetical nucleic acid is replicated in the cell and associates with PrP^{C}, which is thereby converted to PrP^{Sc}. **B:** Models for the conversion of PrP^{C} to PrP^{Sc}. **a)** The refolding model. The conformational change iskinetically controlled, a high activation energy barrier preventing spontaneous conversion at detectable rates. Interaction with exogenously introduced PrP^{Sc} (blue circle) causes PrP^{C} (yellow square) to undergo an induced conformational change to yield PrP^{Sc}. This reaction could be facilitated by an enzyme or chaperone. In the case of certain mutations in PrP^{C}, spontaneous conversion to PrP^{Sc} can occur as a rare event, explaining why familial Creutzfeldt–Jacob disease (CJD) or Gerstmann–Sträussler–Sheinker syndrome (GSS) arise spontaneously, albeit late in life. Sporadic CJD (sCJD) might arise when an extremely rare event (occurring in about one in a million individuals per year) leads to spontaneous conversion of PrP^{C} to PrP^{Sc}. **b)** The seeding model. PrPC (yellow square) and PrP^{Sc} (or a PrP^{Sc}-like molecule; shown as a blue circle) are in equilibrium, with PrP^{C} strongly favoured. PrP^{Sc} is only stabilized when it adds onto a crystal-like seed or aggregate of PrP^{Sc}. Seed formation is rare; however, once a seed is present, monomer addition ensues rapidly. To explain exponential conversion rates, aggregates must be continuously fragmented, generating increasing surfaces for accretion (Weissmann, 2004).

The mechanism of self-propagating alteration of PrP^{C} to pathogenic scrapie form PrP^{Sc} within the proposed protein-only hypothesis is unknown. Two models for the conversion of PrP^{C} to PrP^{Sc} were suggested. The first one, named refolding model, proposes the PrP^{C} is undergoing modification under the influence of a PrP^{Sc} molecule (Prusiner, 1991). The second one, named seeding model suggests that both normal and abnormal PrP isofoms are present in some equilibrium with the strong prevalence of PrP^{C}. This balance is being broken in case the "seeds" of PrP^{Sc} come into the game upon infection (Orgel, 1996). Once the seeds occur, the oligomer formation and modification of PrP^{C} to PrP^{Sc} ensues rapidly.

Although the prion-only hypothesis is broadly accepted in the scientific community, it has certain weaknesses. As it was mentioned above, some strains appeared to be resistance without being infectious, but in some cases the infectivity is being propagated in the absence of detectable PrP resistance to proteolysis (PrP^{res}) (Lasmezas *et al.,* 1997). Scrapie and other TSEs have a different 'strains' characterized by variable incubation periods, clinical features, and neuropathology. They could have distinct abilities to catalyze PrP conversion and could selectively target different brain regions, producing the diversity of clinical symptoms and neuropathological alterations characteristic of prion strains. Small quantities of nucleic acids were detected in infectious samples and PrP^{res} interacts with high affinity with nucleic acids, especially RNA, which could help to catalyze the conversion of PrP^{C} into PrP^{res} *in vitro*. All those controversies together make the prion hypothesis doubtful (Soto and Castillo, 2004).

Putative prion dissemination pathways

Above arguments unambiguously suggest the important role of PrP in prion diseases. The next essential event in the epidemiology of TSE is the infectivity of its agent, i.e. prion. The transmission of a TSE from one species to another is far less efficient than within the same species and even sometimes impossible, which hence resulted in a concept of a "species barrier". However, the events in zoological collections in Great Britain and France lead to conclusion that the "species barrier"-crossing is possible. Animals with diagnosed TSEs were effectively infected with contaminated foodstuff (Sigurdson and Miller, 2003). The possible exception was greater kudu infected by horizontal spread among animals in a manner similar to scrapie and CWD (Kirkwood *et al.,* 1993). Thus, prion disease is mainly acquired through oral infection and then spread from the peripheral to the central nervous system (CNS) (Weissmann, 2004).

The environmental risks of the TSE infection agent have recently being studied. The contaminated soil can become a potential reservoir of TSE infectivity as a result of (i) accidental dispersion from storage plants of meat and bone meal, (ii) incorporation of meat and bone meal in fertilizers, (iii)

spreading of effluents of slaughter-houses, rendering plants and gelatin industry, (iv) possible natural contamination of pasture soils by grazing herds and (v) burial of carcasses of contaminated animals. Environmental sources of contaminated with TSE infection agent (particularly, CWD) represent potential obstacles to control in natural and captive settings. Under experimental conditions, mule deer (*Odocoileus hemionus*) became infected in paddocks containing naturally infected deer, in paddocks where infected deer carcasses were decomposed for approx. 2 years and the paddocks where infected deer had last resided more than 2 years earlier (Miller *et al.,* 2004). The exact mechanisms for CWD transmission in excreta-contaminated paddocks is uncertain, but foraging and soil consumption seems to be most probable, while the fate of PrP in the soil was unclear (Miller *et al.,* 2004).

Earthworms: a putative factor of the dissemination of TSE infectivity in soil?

Soil and soil minerals serve as a reservoir of TSE infectivity (Brown and Gajdusek, 1991). Recent investigation used ovine recPrP and mica (a phyllosilicate, has similar physicochemical surface properties to soil clays) considered as a model of negatively charged mineral surfaces have shown strong adsorption of recPrP to the surface of mica. It was suggested that molecules, which are adsorbed never leave the surface even after a very short residence time at the interface and the mobility of the proteins at the interface was very low. Negative surfaces such as mica are able to concentrate protein, and possibly to change protein conformation (Vasina *et al.,* 2005). Though that seems to be unlikely that interaction of normal prions (PrP^{C}) with soil clay surfaces could induce a change of conformation leading to the pathogenic form of prions (PrP^{Sc}) (Revault *et al.,* 2005). PrP^{Sc} was adsorbed to montmorillonite and kaolinite, quartz, and four whole soil samples and preserve its infectivity (Johnson *et al.,* 2006). Thus, soil micro- and mesoorganisms (soil invertebrates) could greatly affect the distribution or decontamination of PrP.

Among the soil invertebrates, earthworms play an essential role in carbon turnover, soil formation, participating in cellulose degradation and humus accumulation. In the upper soil horizon the earthworms generate mosaic microzone by their activity (Brown, 1995; Devliegher and Verstraete, 1997; Römbke *et al.,* 2005; Tiunov and Kuznetsova, 2000). The earthworms do (i) penetrate soil by burrow activity hence increasing aeration; (ii) transfer soil and organic matter by casting; (iii) humiliate organic material as a first step in organic matter breakdown (including cattle feces in meadows); (iv) change the diversity and improve the activity of the microbial community by selective feeding and provide feces rich in nutrients. Bearing in mind large numbers of these animals in the soil one may consider their significant effect on microbial population of soil.

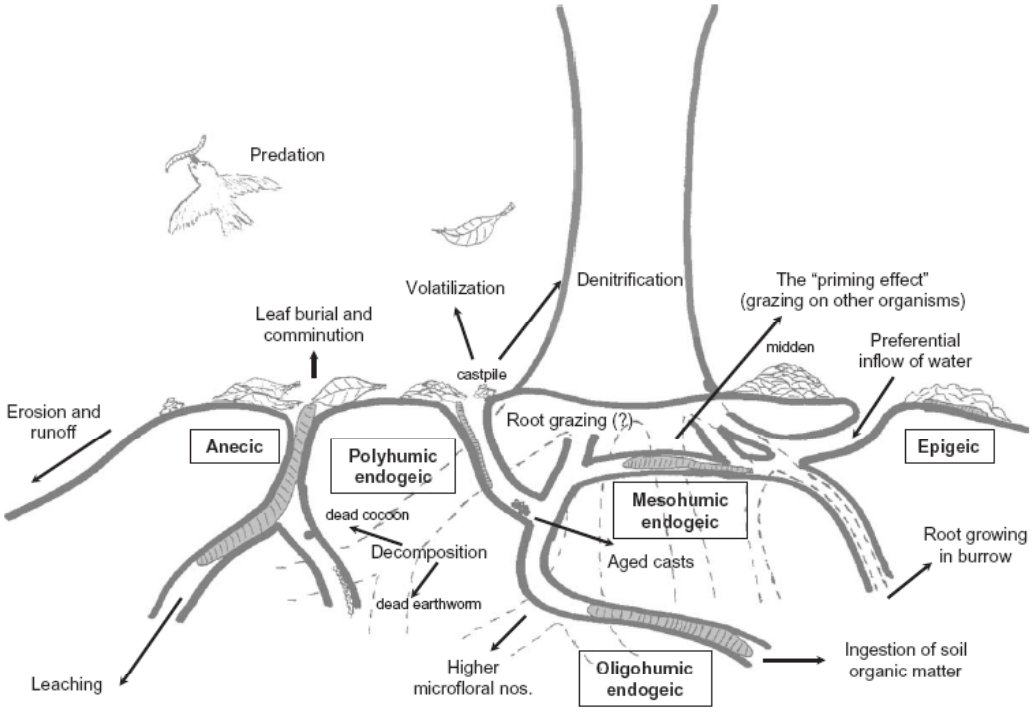


Figure 2. Schematic representation of the functional relationships between earthworms and their external environment (Römbke *et al.,* 2005).

First of all, earthworm gut performs a unique environment subsystem of soil environment. The earthworm gut has stable conditions different from surrounding environment: permanent anoxia, the pool of free amino acids, organic acids, alcohols, sugars and of hydrogen, the products of organic oligo- and polymer degradation (Karsten, and Drake, 1995; Horn *et al.,* 2003). The gut mucosa could be used as an environment and nutrition by microorganisms and gut-derived enzymes of earthworms could affect the microbes on the soil particles (Brown, 1995). On the other hand, the earthworm is migrating in the soil and thus soil (or another substrate as leaf litter or manure composts) flows through earthworm gut being affected by this system.

One aspect of environmental significance of earthworms discovered recently was the extensive N_2O production by microorganisms in the earthworm gut (Karsten and Drake, 1995, 1997; Matthies *et al.,* 1999; Ihssen *et al.,* 2003; Horn *et al.,* 2003, 2006). The numbers of fermentative anaerobes and microbes that used nitrate as a terminal electron acceptor were approximately 2 orders of magnitude higher in the earthworm gut than in the soil from which the earthworms originated. In the gut of the earthworms *Aporrectodea caliginosa* and *Eisenia fetida* the microbial composition was changed towards the increasing numbers of spore non-forming bacteria and decreasing numbers of spore-forming bacteria, which also resulted in enhanced levels of nitrogen fixation (Tereschenko and Naplekova, 2002). A number of novel N_2O-producing species of bacteria from genera *Dechloromonas* (*Betaproteobacteria*), *Flavobacterium* (*Cytophaga-Flavobacteria* group of

Bacteroidetes), and *Paenibacillus* (class *Bacilli*) were isolated from the gut of *Aporrectodea caliginosa* (Horn *et al.,* 2005).

Microorganisms in the earthworm gut and the effect of the gut environment on microbial populations were extensively studied in the past with the classical methods of microbiology. The extensive data on the cultured organisms inhabiting the earthworm-associated ecosystems one should consider with a certain salt grain due to the well-known disadvantages and biases of the plating/culturing techniques. Nevertheless some regularities could be noticed.

The first point, the augmentation with some groups of bacteria, which occurred upon transit through the gut. This phenomenon was described for *L. terrestris* by Daniel and Anderson (1992). Besides, the number of the living bacterial cells estimated by epifluorescence microscopy method, in general correlated with data from plating method (Kristufek *et al.,* 1992).

The ratio of microbes capable of growth under obligatory anaerobic conditions to those capable of growth aerobically was higher in the worm intestine than in the soil (Karsten, Drake, 1995). The total number of platable aerobic and facultatively anaerobic bacteria was $7x10^6$ g^{-1} dry gut content in the foregut, but it increased to $1.6x10^7$ and $2{,}9x10^7$ in the midgut and hindgut of *Lumbricus rubellus*. The increasing number of anaerobic bacteria correlated well to the anaerobic conditions deteced in the worm gut.

Actinomycetes were constantly isolated from the gut of different earthworm species and consdered to be an abundant bacterial group in the microbial community of the earthworm gut. Their number increased in the foregut of *A. caliginosa* (Kristufek *et al.,* 1992). The actinomycetes of genera *Streptomyces* (including *S. diastsatochromogenes*, *S. nogalater*) and *Micromonospora* being a dominant in the gut of the earthworms *L. rubellus* and *Octolasion montanum* produced antibiotics acting against *Bacillus subtilus* and *Sachharomyces serevisiae* (Kristufek *et al.,* 1993). The actinobacteria (*S. lipmanii, S. olivaceus, S. antibioticus*) together with *Vibrio*-type bacteria were considered as pre-dominating organisms in the earthworms *E. lucens* (Contreras, 1980).

Simultaneously, certain bacteria decreased their numbers upon passage: the *Gammaproteobacteria, E. coli* BJ18 and *P. putida* MM1 and MM11, decreased their numbers upon passage, while the transit did not affect *Aeromonas hydrophila* DMU115 or *Enterobacter cloacae* A107 (Pedersen and Hendriksen, 1993).

Plant pathogen fungus *Fusarium oxysporum* was able to survive upon passage through the gut of earthworm *Pheretima* sp. though the earthworms caused a decline of total cell number of the plant pathogen in soil but expanded its distribution in the soil (Toyota and Kimura, 1994). Other authors detected that numbers of micromycetes in the guts of *L. rubellus* and *A. caliginosa* were relatively stable (Kristufek *et al.,* 1992).

Microflora of intestinal guts of *Lumbricus terrestris* and *Octolasion cyaneum* was extensively studied with electron microscopy (Jolly *et al.,* 1993), however no clear message could be taken for. Application of the molecular tools allowed a more accurate estimation of performances of separate microbial groups. Microbial populations in soil and casts of the earthworm *Lumbricus rubellus* were examined through cultivation and 16S rDNA sequence analysis of the clone libraries. The clones related to the *Acidobacteria*, *Cytophagales*, *Chloroflexi*, and *Gammaproteobacteria* were detected in the libraries. Among the isolates, *Aeromonas* spp. were dominating, although these bacteria were not isolated from the soil, besides, the other *Gammaproteobacteria* were found to be quite abundant among clones (49%). The soil isolates were mostly *Actinobacteria* (53%), *Firmicutes* (16%), and *Gammaproteobacteria* (19%). Isolates obtained from the sides of earthworm burrows were not different from soil isolates. Diversity indices for the collections of isolates along with rRNA gene libraries indicated that the species richness and evenness were decreased in the casts compared to their levels in the soil. These results were consistent with a model where a large portion of the microbial population from the soil passes through the gastrointestinal tract of the earthworm remains unchanged, while representatives of some phyla increase their abundance (Furlong *et al.,* 2002).

Filamentous fungi in the gut of *L. terrestris* estimated with fluorescence image analysis were found mainly disrupted before arriving in the intestine. Remaining hyphae in the foregut were completely digested during passage through the gut. Spores of fungi were not detected throughout the study. Numbers of bacteria usually increased from fore- to hindgut. This increase did not correlate to contents of organic material and only partially due to a multiplication of bacterial cells. Numbers of dividing cells accounted in total for approximately 12% of all bacteria, increasing from fore- to hindgut. (Schönholzer *et al.*, 1999). Following estimation of changes of microbial community structure upon passage through the digestive tract of *L. terrestris* showed significantly reduction of bacterial populations belonging to the α-, β- and γ-classes of *Proteobacteria*. Populations of the δ-subdivision of *Proteobacteria* and the *Cytophaga–Flavobacterium* cluster of the *CFB* phylum increased in cast. These results suggest a large impact of passage through the digestive tract of *L. terrestris* on bacterial community structure (Schönholzer *et al.,* 2002).

The work of Egert and colleagues (2004) addressed bacterial and archaeal community structures in soil, gut, and fresh casts of *L. terrestris* using terminal-restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA gene fragments. Ecological indices of community diversity and similarity, calculated from the T-RFLP profiles revealed only small differences between the bacterial and archaeal communities in soil, gut, and fresh casts under both feeding conditions, especially in comparison to other soil-feeding invertebrates. Since no dominant gut-specific OTUs

were detected, the existence of an abundant indigenous earthworm microbial community is in the opinion of the authors rather doubtful, at least in the midgut region of *L. terrestris* (Egert *et. al.,* 2004).

The bacteria associated with the intestine and casts of another earthworm, *L. rubellus,* were examined with 16S rRNA gene clone libraries, and fluorescence *in situ* hybridization (FISH) by Singleton and co-authors (2003). Bacterial libraries constructed from washed earthworm intestine tissues contained several phylotypes, which were rare or absent in the cast libraries. The specific phylotypes present depended on the date of sampling and included representatives of the *Acidobacteria*, *Firmicutes*, *Betaproteobacteria*, and one phylogenetically deep, unclassified group. Juvenile earthworms collected subsequently contained three of the four phylotypes observed in the intestine clone libraries. The *Firmicutes* phylotype was examined by FISH and was found to be a short rod that was represented only a small fraction of the total population of the juvenile samples. These results suggested that the microbial community tightly associated with the intestine was not diverse and was represented rather by *Gammaproteobacteria* (Singleton *et al.,* 2003).

Bacteria were detected also inside the earthworm bodies. They populated the nephridia of earthworms (*L. terrestris*, *A. tuberculata*, *O. lacteum*, and *E. fetida*) and were studied recently with FISH and 16S rDNA sequence analyses. 16S rRNA gene sequences of the symbionts formed a monophyletic cluster within the genus *Acidovorax*. Similarity between symbiont sequences from different host species was 95.5–97.6%, whereas similarity between symbiont sequences from individual animals of the same species was >99%. Bacteria of the genus *Acidovorax* were dominant in the nephridia according to the FISH analysis performed with *Acidovorax*-specific oligonucleotide probe. Thus, these bacteria were suggested to be an earthworm symbiont, which could play a role in protein degradation during nitrogen excretion by earthworms (Schramm *et. al.* 2003). Davidson and Stahl (2006) who performed curing experiments, FISH analysis with *Acidovorax*-specific probes, and 16S rRNA gene sequence analysis, provided the evidence, that the egg capsules of *E. fetida* contain high numbers of the bacterial symbiont and that nephridia of juveniles are colonized during development within the egg capsule.

One of the major problems of previous studies was that some of the authors have used analyses based on the direct enumeration (e.g. through FISH) alone (Schönholzer *et al.,* 2002, Fisher *et al.,* 1995), the others used solely the PCR amplification of 16S rDNA and sequencing analysis of the derived libraries (Furlong *et al.,* 2002, Egert *et. al.,* 2004). However, the higher stability of DNA in environments in many cases does not allow to adequately concluding on the microbial community composition. Still, compared to the well-studied termite gut microflora, the intestinal microbiota of the earthworm remains poorly understood. There is no data available about regularity of alteration

of soil microbial community upon its passage through the gut of different earthworm species and the main factors (microbial community of substratum or earthworm gut environment) affecting these alterations.

To sum up, currently there is no solid data on the major patterns (if any) on the alterations of microbial community from initial substratum passing through the gut. This basic knowledge is absolutely necessary if one aims at the analysis of the worm-associated microbiome as a potential factor influencing PrP in the soil. Consequently, no studies were conducted up to date to determine the potential of the microorganisms, both, autochtonous or passing through the gut, of the worm (or other soil invertebrates), to influence the fate of infectious agents, such as PrP.

The present work was a part of the European Union Project 'Biotic and Abiotic Mechanisms of TSE Infectivity Retention and Dissemination in Soil' (QLRT-2001-02493) especially dedicated to study the possible pathways of the prion dissemination in soil or soil-associated systems. A number of ecological and epidemiological studies aimed at the understanding the mechanisms of interaction of model prion (ovine recombinant prion, recPrP) with soil clay minerals and organic compaunds (humus), migration of TSE infectivity through the soil column; effect of soil microorganisms and microbes associated with soil invertebrates on the prion exposed, and the role of necrophagous insects in the dissemination of TSE infectivity from soil-buried carcasses.

Objectives of the study

The aim of this investigation was to estimate the potential role of microorganisms associated with earthworm intestine in the rPrP fate in the soil. Two major tasks of the present study were:

- To characterize the microbial community in the earthworm gut: (i) to monitor the population changes of different taxonomic groups in substrata passing through the earthworms guts and define their common regularities; (ii) to estimate the composition of microbial communities and (iii) to define the putative gut-associated microorganisms.
- To determine the potential activity of microorganisms for recPrP proteolysis: (i) proteolytic activity of pure bacterial and fungal isolates; (ii) proteolysis of recPrP by soil microbial community and upon passage through the earthworm gut by intestine-augmented/stimulated microbes.

2. Materials and Methods

2.1 Sampling and general experimental design

Three earthworm species were used in the study, *Lumbricus terrestris* and *Aporrectodea caliginosa,* typically populating soddy-podzolic arable soil and *Eisenia fetida* inhabiting horse manure compost. Soddy-podzolic soil and the earthworms were collected from the top ploughed horizon (0-20 cm) under crop rotation at Ecological Soil Station of Moscow Lomonosov State University (Solnechnogorskiy district, Moscow region, Russia). Horse manure compost and earthworms *E. fetida* were taken at Chashnikovo Biological Station (Solnechnogorskiy district, Moscow region, Russia).

Three groups of earthworms were analyzed: (1) three earthworm species (*L. terrestris* represented by 3 animals, 6 individual animals of *A. caliginosa* and 5 of *E. fetida*) with respective substrata) were collected at the autumn 2002; (2) 10 earthworms *A. caliginosa* and soil were taken at the spring 2003; and (3) 30 exemplars of *L. terrestris* and 50 exemplars of *A. caliginosa* with the soil were collected in the autumn 2004. Groups (1) and (2) were used in the preliminary experiments for studying the most common regularity in changes of microbial community passing through the gut of the earthworms. The group (3) was used for more detailed investigation of both intestine bacterial populations and for rPrP proteolytic activity of microbial populations from the soil and earthworm sources. Different species of the animals from the first group were held together to estimate the effect of individual features of species on changes of soil bacterial community upon passage through the worm guts. The earthworms from the third group were kept separately each from other. The first two groups were kept for two weeks at 15° C, while the third group was kept for three month before the experiment at same temperature. During this time earthworms were fed with nonsterile oak leaf litter.

Pure microbial cultures were also using for 16S and 18S rRNA gene sequence analysis and we performed the identification of the pure cultures of microorganisms isolated by our colleagues from the Department of Soil Biology of the Moscow Lomonosov State University (Moscow, Russia) from the earthworms, soil and worm excrements. The isolates were consequently scored with various proteolytic assays.

The culture-independent rRNA-based techniques (FISH and RT-PCR) were applied for investigating biodiversity of the soil, compost, earthworm intestine, and casts. Taxon-specific SSCP analysis was performed for a rapid study of microbial communities. Clone libraries were

constructed to roughly estimate bacterial composition and FISH analysis was applied for a direct enumeration of microorganisms from different taxonomic groups.

2.2 Fluorescence *in situ* Hybridization (FISH)

2.2.1 FISH with soil, intestine, and casts samples

Isolation of microbial cells from environmental samples

Substrates (soddy-podzolic soil and compost) 2 g each were fixed overnight at 4° C in 10 ml of freshly prepared 4 % paraformaldehyde/PBS (137 mM NaCl; 2,7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4) solution. Then samples were homogenized with vortexing for 5 min. and centrifuged at 800 g for 1 min. Microorganisms were collected from supernatant by separation cells from soil particles via Nycodenz® gradient as described previously (Berry *et al*., 2003). Cells were washed twice with PBS and stored in PBS/ethanol (1/1) solution at –20° C.

Casts were collected by keeping earthworms on the wet sterile filter paper for 6 hours at 15° C and then earthworms were placed back into the soil/compost substratum. Collected fresh casts were fixed and treated as the soil samples above.

The guts were dissected, the gut content and empty guts were fixed separately in 4% paraformaldehyde in PBS overnight at 4° C. Microorganisms from the gut walls were washed off with PBS (1,5 ml) for 2 min and vortexing in Matrix tube (Fast RNA Spin Kit for soil, Qbiogene). Big particles of gut were excluded from suspension by centrifugation for 1 min at 200 g. Cells from supernatant were collected with centrifugation for 4 min at 10000 g and stored in PBS/ethanol (1/1 vol/vol) solution at –20° C.

Fluorescence *in situ* Hybridization procedure

The aliquots (5 or 10 µl) of the samples were hybridized with Cy3-labeled oligonucleotide probes EUB338 (5'-GCTGCCTCCCGTAGGAGT-3') to specifically detect most of *Bacteria* (Amann *et. al*., 1990); ALF968 (5'-GGTAAGGTTCTGCGCGTT-3') for *Alphaproteobacteria* excluding *Rickettsiales* (Neef, 1997); BET42a (5'-GCCTTCCCACTTCGTTT-3') for *Betaproteobacteria* and GAM42a (5'-GCCTTCCCACATCGTTT-3') for *Gammaproteobacteria* (Manz *et al.,* 1992); DELTA495a (5'-AGTTAGCCGGTGCTTCCT-3') for most of *Deltaproteobacteria* (Loy *et. al*., 2002); CF319a (5'-CCGTMTTACCGCGGCTGCTGGCA-3') for *Cytophaga-Flavobacterium*-group of the *Bacteroidetes* (Manz *et al.,* 1996); EUB3338 II (5'-GCAGCCACCCGTAGGTGT-3') for Planctomycetes and EUB3338 III (5'- CTGCCACCCGTAGGTGT-3') for *Verrucomicrobia* (Daims H. *et al.,* 1999); LGC354A (5'-TGGAAGATTCCCTACTGC-3') for *Firmicutes* (Gram-

positive bacteria with low G+C content) (Meier *et. al.*, 1999); HGC69A (5-TATAGTTACCACCGCCGT-3) for *Actinobacteria* (Gram-positive bacteria with high G+C content) (Roller *et. al*., 1994); PF2 (5'-CTCTGGCTTCACCCTATTC-3') for all yeasts (Kempf *et. al*., 2000); ARCH915 (5'-GTGCTCCCCCGCCAATTCCT-3') for *Archaea* (Stahl and Amann, 1991). Details of these oligonucleotide probes are available at ProbeBase URL, http://www.microbial-ecology.net/probebase/ (Loy *et. al.,* 2003).

2.2.2 Design of group-specific nucleotide probe and the FISH with the earthworm tissues

The specific nucleotide probe was designed to detect *Mollicutes*-like organisms (MLO) whose sequences were found in the clone libraries from earthworm gut content and casts. These sequences were aligned together with other related sequences including those from class *Mollicutes* available from the GenBank database (Benson *et al.,* 2003) using ClustalW software (Thompson *et al.,* 1994). Alignment files were processed with BioEdit software to design primer specific for target bacterial group. Such specific oligonucleotide probe was named LUM1225 (5'-GCTTACTGTCACCAGTTT-3') (corresponding to its position in the 16S rRNA gene in *M. pulmonis* (AF125582)). The specificity of the primers was checked against the RDPII database (Cole *et al.,* 2005) using Probe Match software. The nucleotide probe labeled with 5'-AlexaFluor546 was synthesized and delivered by Invitrogen (Germany).

Preparation of the tissues and hybridization were done according to the previously published data (Boye *et al.,* 2001; Amand *et al*., 2005). The earthworms were narcotized with ethyl ester. Middle body parts were dissected and fixed in the 10% buffered formalin for 24 hrs at 4° C, with following washing in PBS. Tissues were embedded in paraffin, cut into 10 µm-thick sections, and mounted on slides prepared with silane (Sigma-Aldrich). The tissue sections were dewaxed in 100% xylene for 10 min and washed in absolute ethanol. All sections were allowed to air-dry before hybridization. Hybridization was performed in 10µl of hybridization buffer (900 mM NaCl, 20 mM Tris (pH 8,0), 0,01% SDS, and 35% formamide) with final concentration of probe 5 ng/µl. The slides were incubated at 44° C for 6h in humid chamber. After hybridization the slides were washed with the buffer consisted of 225 mM NaCl, 5 mM EDTA, 0,01% SDS, and 20 mM Tris (pH 8,0) for 20 min at 46° C. The slides were rinsed with distilled water, air-dried in the dark, and finally amended with Citifluor antifading agent. The slides were examined with Axioskop 40 epifluorescence microscope (Zeiss, Germany) and Axiovert 100 TV laser confocal microscope (Zeiss, Germany).

2.3 rRNA and rRNA gene amplifications

2.3.1 Constructing of 16S rRNA clone libraries

Total DNA/RNA isolation and reverse transcription

Total DNA was extracted from the soil and cast samples with FastDNA SPIN Kit for Soil (Qbiogene, Germany) quantified spectrophotometrically and was directly utilized as the template for PCR.

Total RNA was extracted from the samples with the Fast RNA Spin Blue Kit (for soil) (Qbiogene, Germany). Quantification of isolated total RNA was also done with BioPhotometer (Eppendorf, Germany). RNA was treated with DNase I (Invitrogen, Germany) according to the manufacturers protocol. The amount of RNA used for reverse transcription was 1µg per reaction (20 µl) for each sample. Single-strand DNA was obtained using Super Script™ First Strand Synthesis System for RT-PCR (Invitrogen, Germany) with the universal primer for 16S rRNA R1492 (5'-CGGYTACCTTGTTACGACTT-3').

PCR-amplification

Amplification of single strand DNA obtained with reverse transcription was done with 16S rDNA specific primers F530 (5'-TCCGTGCCAGCAGCCGCCG-3') and R1492. Amplification was done in 20µl reaction with *Taq* DNA Polymerase, recombinant (Invitrogen, Gemany) and original reagents according to the basic PCR protocol. Amount of ssDNA template was 30-50 ng/reaction. Reaction mixtures were subjected to the following thermal cycling parameters: 30 cycles of 96° C for 1 min, 45° C for 1 min, 72° C for 2 min, followed by a final extension at 72° C for 10 min. Achieved PCR products were purified by QIAEX II Gel Extraction Kit (QIAGEN, Germany) from the 0,8% agarose gel.

Constructing of clone libraries

The purified PCR products were ligated into plasmid vector pCRII-TOPO with following transformation into the electrocompetent cells *E. coli* (TOPO 10) by electroporation (U, 1,8 kV; R, 200 Om) (TOPO TA Cloning kit, Invitrogen, Germany). Colonies were blue/white screened on LB agar with 50µg/ml kanamycin and 25µl/ml X-Gal (Promega, Germany). Randomly chosen clones were transferred into 96-well plates and further analyzed.

Sequencing of cloned 16 rDNA and phylogenetic analysis

Bacterial clones were grown in the 96-well microtiter plates with 100 µl of LB medium with kanamycin (50 µg/ml) at 37° C overnight. Bacteria were pelleted by centrifugation (1000 g for 3 min at 4° C), washed once with 1x PBS and resuspended in the PCR-lysis solution A without proteinase K (67 mM Tris-Cl (pH 8,8); 16 mM NH_4SO_4; 5 µM β-mercaptoethanol; 6,7 mM $MgCl_2$; 6,7 µM EDTA (pH 8,0) (Sambrook, Russel, 2002) and heated at 95° C for 10 min.

PCR amplification was done with above bacterial lysate (1µl), *Taq* DNA Polymerase, recombinant (Invitrogen) and original reagents according to the basic PCR protocol using primers M13 forward (5'-GACGTTGTAAAACGACGGCCAG-3') and M13 reverse (5'-GAGGAAACAGCTATGACCATG-3') in 20µl of final volume with thermal/time conditions described above. PCR products were purified with MinElute 96 UF PCR Purification Kit (Qiagen, Germany).

The monodirectional sequencing was proceeded with reverse primer R1492 according to the protocol for BigDye Terminator v1.1 Cycle Sequencing Kit from Applied Biosystems (USA): Ready Reaction Premix (2,5x) 4µl; BigDye Sequencing Buffer (5,0x) 2µl; primer 10pmol; clear PCR product 1µl; deionized water up to 20µl) followed by 25 cycles of 96° C for 20 s, 50° C for 20 s, 60° C for 240 s, followed by at 4° C hold.

Obtained sequences were analyzed against the GenBank database using BLAST alignment software (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1997).

2.3.2 Taxon-specific Single Strand Conformation Polymorphism (SSCP) analysis

Design of taxon-specific 16S rRNA gene primers.

Sequences (minimum of 1300 bp long) of described bacterial species of *Proteobacteria* (α, β, γ, δ-classes), *CFB* group, *Planctomycetes*, *Verrucomicrobia*, *Actinobacteria* (HGC), and class *Bacilli* available from the GenBank database (Benson *et al.,* 2003) and related to those detected with culturing and RT-PCR approaches were selected and aligned with outgroups sequences using ClustalW software (Thompson *et al.,* 1994). Alignment files were processed with BioEdit software to design primers, which were supposed to be consent with taxon-conserved regions, 18-25 bp-long and be situated between 400 and 500 each from other. Specificity of the primers was checked with PrimeRose software (available at the URL: http://www.cf.ac.uk/biosi/research/biosoft/Primrose/index.html) against constructed databases, including 16S rDNA sequences of isolated bacterial strains and RT-PCR obtained from the analysed ecosystems. Additionally primers were screened against the RDPII database (Cole *et al.,*

2005) using Probe Match software. Less specific primer in the set carried phosphate group on 5'-terminus.

Specificity of the primers for annealing was determined using temperature gradient PCR in thermocycler Eppendorf 5341 (Germany). Reactions were performed in 20μl of final volume using recombinant *Taq* DNA Polymerase (Qiagen, Germany) and original reagents according to the basic PCR protocol. Bacteria were lysed in PCR-lysis solution A without proteinase K (67 mM Tris-Cl (pH 8,8); 16 mM NH_4SO_4; 5 μM β-mercaptoethanol; 6,7 mM $MgCl_2$; 6,7 μM EDTA (pH 8,0) (Sambrook, Russel, 2002) and heated at 95° C for 5 min were used as DNA template. 16S cDNA was used as template for *Verrucomicrobia*, *Planctomycetes* and *Myxococcales*.

Reaction mixtures were subjected to the following thermal cycling parameters: 30 cycles of denaturation at 96° C for 1 min, annealing with temperature gradient (first frame at 45-55°, and second frame at 55-65° C) for 1 min, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. Outgrouped bacterial species were used as a negative control. The quality of PCR products was verified through 2% agarose gel electrophoresis.

Table 1. Sets of taxon-specific SSCP primers and their annealing temperatures

Target group	Primer Name[1]	Primer Sequence	Annealing[3] T, °C	Expected fragment, bp[3]
α -*proteobacteria*	ALF F822p[2]	5'-CCACGCCGTAAACKATGA-3'	50	412
	ALF R1234	5'-CSYGTAAGGGCCATGAGG-3'		
β -*proteobacteria*	BET F750p	5'-GACGCTCAKGCACGAAAGCGT-3'	45	477
	BET R1227	5'-TGACGTGTGWAGCCCCACCYA-3'		
γ -*proteobacteria*	GAM F538p	5'-RAGGGTGCAAGCGTTAAT-3'	50	503
	GAM R1041	5'-YNNNGTTCCCGAAGGC-3'		
Myxococcales	DEL F972p	5'-CGCAGAACCTTACCTGGK-3'	48	462
(δ-*proteobacteria*)	DEL R1434	5'-GACTTCTGGAGCAAYYG-3'		
CFB	CFB F522	5'-TYAYTGGGTTTAAAGGGT-3'	50	417
	CFB R939p	5'-TAAGGTTCCTCGCGTANCA-3'		
Verrucomicrobia	VER F901p	5'-AGCGGTGGAGTATGTGGC-3'	48	485
	VER R1209	5'-GCATTGTAGTACGTGTGC-3'		
Planctomycetes	PLA F949p	5'-GCGMARAACCTTATCC-3'	48	459
	PLA R1408	5'-CCNCNCTTTSGTGGCT-3'		
Bacilli	BAC F348p	5'-CAGCAGTAGGGAATCTTC-3'	50	485
(*Firmicutes*)	BAC R833	5'-ATGARTGCTARGTGTTAG-3'		

[1]positions in the target groups: *Alphaproteobacteria* (*B. diminuta* X87274); *Betaproteobacteria* (*A. faecalis* AF155147); *Gammaproteobacteria* (*E. coli* NC004431); δ-proteobacteria (*M. fulvus* AJ233917); CFB (*F. jonsoniae* AB078043); *Verrucomicrobia* (*V. spinosum* X90515); *Planctomycetes* (*P. limnophilus* X62911); *Bacilli* (*B. subtilis* AY030331); [2] p – indicates the phosphate group;
[3] annealing temperature calculated for the primer set;

SSCP: total DNA/RNA isolation and PCR

Total DNA and RNA were extracted from the soil and cast samples as it was described above. Amplification with taxon-specific primers (RNA was previously undergone reverse transcription) was performed at annealing temperature illustrated in Table 1. Additionally, universal primers Com1 (5'-CAGCAGCCGCGGTAATAC-3') and Com2-Ph (5'-CCGTCAATTCCTTTGAGTTT-3') were used (Schweiger and Tebbe, 1998). Amplification was done with serial dilutions of template. Obtained PCR products were purified with QIAEX II Gel Extraction Kit (QIAGEN, Germany) from the 2% agarose gel.

Preparation of ssDNA and gel electrophoresis

Pure PCR products were treated with λ–exonuclease (Fermentas, Germany) and consequent SSCP analysis was performed as it was previously described (Schwieger and Tebbe, 1998) on 21 cm-long 0,6X MDE gels (Cambrex, Germany) with glycerol (5%). The electrophoresis was performed in a Pharmacia Multiphor II apparatus (Pharmacia, Germany) at 400 V for 14 hours at 20° C. Gels were silver stained (Bassam *et al.* 1991) and dried at room temperature.

Isolation and PCR amplification of DNA fragments from polyacrylamide gels.

Single bands detected in polyacrylamide gels after silver staining were cut out with disposable scalpel blades. Gel slices were transferred to 96-well microtiter plate containing 20 µl of elution buffer (10mM Tris-Base, 5mM KCl, 1,5mM MgCl x $6H_2O$, 0.1% Triton X100, pH 9,0) and boiled at 95° C for 10 min. Reamplification with corresponding primers was done under conditions described above with 1 µl supernatant as a template after spinning down the samples. PCR products were purified with MinElute 96 UF PCR Purification Kit (Qiagen, Germany).

Sequencing of 16 rDNA

The monodirectional sequencing was done with corresponding downstream primers according to the protocol for BigDye Terminator v1.1 Cycle Sequencing Kit from Applied Biosystems (USA): Ready Reaction Premix (2,5x) 4µl; BigDye Sequencing Buffer (5,0x) 2µl; primer 10 pmol; clear

PCR product 1µl; deionized water up to 20 µl) followed by 25 cycles of 96° C for 20 s, 50° C for 20 s, 60° C for 240 s, followed by 10 min-hold at 4° C.

In case the sequencing reaction was unclear, the PCR product was cloned with TOPO TA Cloning Kit (Invitrogen, Germany) and sequenced from the pCRII plasmid with M13F or M13R oligonucleotides as described above.

Obtained sequences were analysed using BLAST alignment software (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1997), Ribosomal Database Project II (RDP) (http://rdp.cme.msu.edu/html/) (Cole *et al*., 2005).

Phylogenetic analysis

Phylogenetic analysis was then performed using the program package Phylip (Felsenstein, 2001) as described by Yakimov and colleagues (2005), and ClustalW online tool (http://www.ebi.ac.uk/clustalw/index.html) (Thompson *et al*., 1994) with sequences loaded from NCBI database (http://www.ncbi.nlm.nih.gov/) (Altschul *et al*., 1997).

Chimera checking and constructing of phylogenetic trees

All cloned insertions with identity less than 95% to already described organisms were analyzed with CHIMERA_CHECK program at the Ribosomal Database Project II (RDP-II) http://35.8.164.52/cgis/chimera.cgi?su=SSU (Cole *et al.,* 2005). The sequences suspected to be chimeric were excluded from analysis.

2.4 Isolation and identification of pure microbial cultures

Isolation of bacteria and fungi with serial plate dilution method

Bacteria and fungi were isolated from soil, compost, gut, and excrements of the earthworms on the glucose-peptone-yeast agar (glucose, 1 g; peptone, 2 g; yeast extract, 1 g; casein hydrolyzate, 1g; KH_2PO_4, 0,5 g; K_2HPO_4, 0,5, g; Difco agar, 15 g; distilled H_2O up to 1000 ml; pH 7,2). Fungal growth was inhibited by nystatin or cycloheximide and bacterial growth was limited by adding the streptomycin sulfate. The isolation of microorganisms was done by plate dilution method and by placing of particles of soil, gut, and excrements directly on the surface of the solid medium. For homogenization of samples and desorbing of microorganisms from mineral/organic particles the homogenizer DIAX 900 (Heidolph) was used. Fifty mg of dry compost or excrements or freshly obtained gut tissue (dry weight around 10-50 mg) was placed into 500 ml of sterile tap water (for

bacteria) or into 10 ml for fungi. The samples were homogenized at 8000 rpm for 30 sec in 10 ml of sterile water. Aliquots (20-400 µl) were spreaded by spatula on the surface of the agar medium. The Petri dishes were incubated for 10-28 days at room temperature (18-20° C).

Microbial strains in several replicates were isolated from each group of colonies that had similar cultural and morphological features on the slants with same medium (for fungi also – on malt-agar) and maintained at 4°C. For fungi, relative abundance of isolates of different species/genera from total isolates from gut or soil on the plates was calculated.

Identification of microbial isolates

The isolates were identified by the sequence analysis of 16S rRNA (bacteria) and the ribosomal intergenic space D1-D2 region (fungi).

Preparing cultures for the PCR amplification

Bacteria were grown in 96-well microtiter plates with 100µl of LB medium (tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g; distilled H_2O up to 1000 ml) at 30° C for 2 days. Consequently the cells were pelleted by centrifugation (Heraeus Sepatech Omnifuge 2,0 RS; 500g at 10^0 C for 15min) and washed once with PBS (NaCl, 137 mM; KCl, 2,7 mM; Na_2HPO_4, 10 mM; KH_2PO_4, 2 mM). Cells were resuspended and boiled in 100µl of PCR-lysis solution A (Tris-Cl (pH 8,8), 67 mM; NH_4SO_4, 16 mM; β-mercaptoethanol, 5 µM; $MgCl_2$, 6,7 mM; EDTA (pH 8,0), 6,7 µM; SDS, 1,7 µM) (Sambrook, Russel, 2002) for 10 min.

Fungal isolates were grown in 96 well-microtiter plates with 50µl of Czapek medium (sucrose, 30,0 g; KNO_3, 2,0 g; K_2HPO_4, 1,0 g; $MgSO_4*7H_2O$, 0,5 g; KCl, 0,5 g; $FeSO_4*7H_2O$, 0,01 g; yeast extract, 2,0 g; peptone, 5,0 g; distilled H_2O up to 1000 ml) in each well, at 30° C for 2 days. Fungal biomass was collected with centrifugation as described above, washed with PBS and treated with lyticase in buffer Y (sorbitol, 1M; EDTA 0,1M; pH 7,4) at 37° C for 30 min. Then biomass was washed with PBS from buffer Y and lyticase and resuspended in 100µl of PCR-lysis buffer A with proteinase K, incubated at 55° C for 2 hrs, and boiled at 95° C for 10 min.

PCR amplification of the 16S rRNA genes of the isolates

Reaction mixes were set up in a PCR hood in a room separated from that used to extract DNA and the amplification and post-PCR room in order to minimize contamination. Reaction mixes (total, 20 µl) were set up as follows: deionized H_2O 6 µl; Q-solution 4 µl; 20 mM solution of four dNTPs (pH 8,0) 4µl; PCR Buffer (10x; contains 15 mM $MgCl_2$) 2,0 µl; 1,0 U of *Taq* DNA polymerase (Amplitaq; Perkin Elmer); $6*10^{-3}$ nmol each primers of the 16S rDNA (for bacteria) primers F27

forward (5'-AGAGTTTGATCMTGGCTCAG-3') and R1492 reverse (5'-TACGGYTACCTTGTTACGACTT-3') and 18S rDNA (for fungi) primer NL-1 forward (5'-GCATATCAATAAGCGGAGGAAAA-3') and NL-4 reverse (5'-GGTCCGTGTTTCAAGACGG-3') and 1 µl of DNA template.

The reaction mixtures were subjected to the following thermal cycling parameters in a Perkin Elmer 9600 thermocycler: 30 cycles of 96 °C for 1 min, 45 °C for 1 min, 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. After amplification the aliquots (4 µl) were removed from each reaction mixture and examined by electrophoresis (150 V, 25 min) in gels composed of 0,8 % (w/v) agarose (Gibco, UK) in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3), stained with ethidium bromide (5 µg/100 ml). Gels were visualized under UV illumination using a gel image analysis system (VVP Products, UK).

Sequencing of amplicons and analysis of sequence data

PCR products were cleaned by MinElute 96 UF PCR purification kit (QIAGEN, Germany) prior to sequencing, particularly to remove dNTPs, polymerases, salts, and primers. The monodirectional sequencing was proceeded with one corresponding primer according to the protocol for BigDye Terminator v1.1 Cycle Sequencing Kit from Applied Biosystems (Ready Reaction Premix (2,5x) 4µl; BigDye Sequencing Buffer (5,0x) 2µl; primer 10pmol; clear PCR product 2µl; deionized water up to 20µl) followed by 25 cycles of 96 °C for 20 s, 50 °C for 20 s, 60 °C for 240 s, followed by a 4 °C hold. The resulted 16S and 18S rDNA sequences were compared with those stored in the GenBank Data system using BLAST alignment software (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1997), and Ribosomal Database Project II (RDP) (http://rdp.cme.msu.edu/html/) (Cole *et al*., 2005).

2.5 RecPrP proteolysis

2.5.1 Recombinant protein synthesis

Ovine ARQ genetic variant of the prion protein (94% homology with the bovine PrP (Rezaei *et al.,* 2002) used for the experiments was synthesized and delivered by our colleagues Dr. Human Rezaei and Mrs. Peggy Rigou from INRA (Institut National de la Recherche Agronomique, Unité de Virologie et Immunologie Moléculaires, Jouy-en-Josas, France). The recombinant PrP (without GPI; cloned in pET 22+) was expressed in inclusion bodies in *E. coli* BL21 DE3 strain according to the previous protocol (Rezaei *et al.,* 2000). After lysis, sonication and solubilization of the inclusion

bodies in urea, purification of the protein was performed on a Ni Sepharose column using the propensity of the N-terminal octarepeat region to chelate transition metals. Refolding of the protein was achieved on the column by heterogeneous phase renaturation simultaneously to purification, favoring intra-molecular rather than inter-molecular disulfide bond formation. After elution by 1 M imidazole, the purified protein was recovered in the desired buffer by passage on a G25 desalting column. This procedure leads to a high degree of purification, as checked by SDS-PAGE and Western Blot and to a strictly monomeric alpha-rich protein.

2.5.2 RecPrP proteolytic assay

2.5.2.1 PrP proteolytic assay of pure isolates

Among more than 1500 bacterial and 400 fungal cultures isolated from the soddy-podzolic soil, horse manure compost, gut content and cast of earthworm (*Aporrectodea caliginosa, Lumbricus terrestris* and *Eisenia fetida*) randomly chosen strains (1 strain from each specie) were investigated for rPrP proteolysis. Medium M9 (Sambrook, Russel, 2002) (11µl) with minor modification (without glucose; peptone – 0,5 g/l; gelatin – 0,5 g/l) was inoculated with pure microbial cultures and 40 ng of recPrP. After 3 day incubating recPrP digestion was checked by Western Blot analysis.

2.5.2.2 Effect of earthworms and gut microbiota on recPrP retaining

Extraction of recPrP from the soddy-podzolic soil and earthworm cast

In the preliminary experiments, two protocols were applied to extract recPrP (100 ng) from soil (1 g).

The first method, elaborated by Dr. Robert Somerville, (Institute of Animal Health, Neuropathogenesis Unit, University of Edinburgh, United Kingdom) proposed to treat 1 g recPrP-contaminated soil/sand sample with 1 ml of the extraction buffer (100 mM Na_2HPO_4-1% sarkosyl). Samples were thoroughly mixed on a vortex mixer and were attached to a horizontal shaker for vigorous shaking at 600 rpm for 2 h. After 2 h, samples were mixed briefly on the vortex mixer, and then spun down at 6000 g for 15 min. The supernatant was then carefully poured off into another 2 ml tube, and a sub-sample (10µL) was taken for analysis by SDS-PAGE and Western blotting.

The second method was based on the electroelution of recPrP from the soil (Rigou *et al.,* 2006). Negatively charged recPrP protein was extracted from soil in an assembly of used tubes filled with acrylamide gel flanked with agarose plugs and covered at the edges with a dialysis membrane. The

tubes were imbedded into the agarose stacked on the tray for a standard horizontal electrophoresis unit. The protein eluted in this device was subjected to Western blot analysis.

The mixture of protease Inhibitor Cocktails Sets (II+III) (Calbiochem) (Tab. 2) was added into the control samples (20 µl/g soil) before extraction with both protocols.

Table 2. Composition of Protease Inhibitor Cocktail Sets (Calbiochem)

Compound	Target protease	Protease Inhibitor Cocktail Set II	Protease Inhibitor Cocktail Set III
AEBSF, hydrochloride	Serine protease	+	+
Bestatine	Aminopeptidase B and Leucine Aminopeptidase	+	+
E-64, Protease inhibitor	Cysteine protease	+	+
EDTA, disodium	Metalloproteases	+	–
Pepstatine A	Aspartic protease	+	+
Aprotinin	Trypsin, chymotrypsin, coagulation factors involved in the prephase of blood clotting tissue and leukocytic proteinases, kallikrein, plasmin	–	+
Leupeptine, hemisulfate	Trypsin-like proteases and cysteine proteases	–	+

Aqueous extracts assay

The aqueous extracts were prepared from the soil and earthworm cast to determine enzymatic proteolytic activity of the water phase. Samples (approx. 0,5 g) were homogenized by vortex in water (50% w/v) and centrifuged at 11000 g for 5 min. Supernatant was filtered through the membrane filter (0,22 µm, Millipore, USA) into the clean tube. General amount of proteins in the solutions were determined with the Bradford dye-binding Kit (Bio-Rad, USA) as described above.

Protease Inhibitor Cocktail Sets II (recommended for use with bacterial cell extracts) and III (recommended for use with mammalian cell and tissue extracts) (Calbiochem) were used to determine the role of proteases derived from bacteria and eucarya in unspecific and recPrP proteolytic activity (Tab. 2). There were in total four experimental setups for aqueous extract with

recPrP: (1) without Inhibitor Cocktails; (2) with Inhibitor Cocktail Sets (II+III); (3) with Inhibitor Cocktail Set II; (4) with Inhibitor Cocktail Set III. Protease Inhibitors (1µl of each cocktails and their equal (vol./vol.) mixture (sets II+III)) were added to correspond setup.

Unspecific proteolytic activity of aqueous extracts was checked with Protease Screening^{TM} Kit (GenoTech, USA) at 15° C for 12 hrs.

RecPrP proteolysis was performed in 12 µl of aqueous extract suspension (total reaction volume) included 40 ng of recPrP and approximately equal amount of proteins in each sample. Sampling was done after 0,2; 1; 2, 6 and 12 hrs as well as after 1, 2, 3, 4, and 6 days of incubation at 15° C. Total sample volume was analyzed with Western Blot analysis for remained PrP amount.

Control digestion of recPrP (40 ng in 12 µl of total reaction volume) was done with modified trypsin (0,8 ng/µl) in the supplemented buffer (New England Biolabs, Germany).

2.5.2.3 Western blot analysis

Western blot analysis was performed as described previously (Thackray *et al.,* 2004). The total sample volume from each experiment was loaded on the SDS-PAGE (12% vol/vol) and after electrophoresis subsequently blotted to the nitrocellulose membranes (PerkinElmer) in transfer buffer (25 mM Tris, 192 mM glycine, 10% isopropanol) at 400 mA (constant) for 1 h as was previously described (Baron *et al.,* 1999) with blotter (Peqlab, Germany). Membranes were blocked with TBS-T (10 mM Tris-Cl pH 7.8, 100 mM NaCl, 0.05 % Tween 20) with 5 % skimmed milk (Thackray *et al.,* 2004) and subsequently incubated with primary mouse antibodies PrP248 for N-terminus (residue numbers 60-95 according to ovine sequence) and VRQ14 for the interhelix loop (residue numbers 194–199 according to ovine sequence) (Rezaei *et al.,* 2005) at the concentration 5 µg/ml for 1 h at room temperature (Fig. 3).

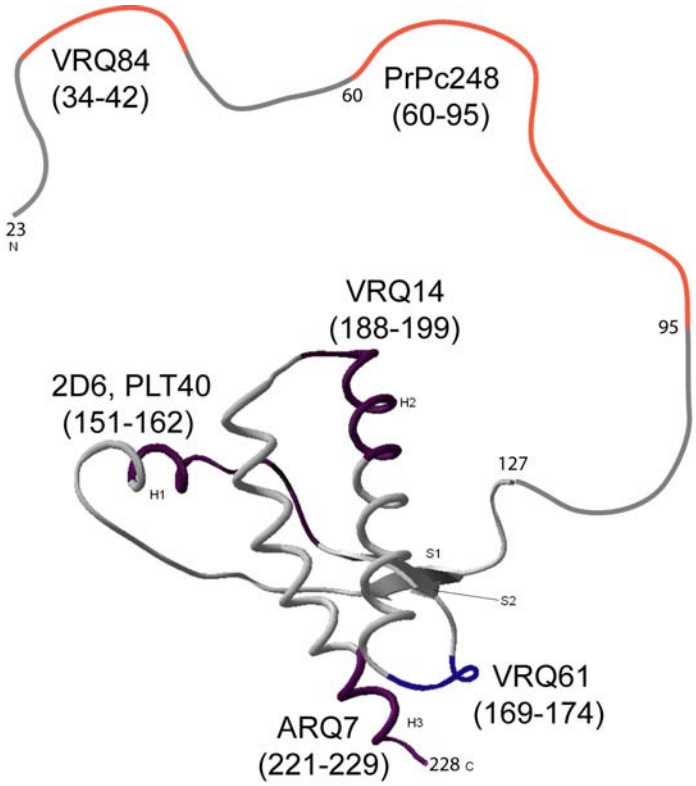


Figure 3. Localization of antibody epitopes on the 3D structure of ovine recPrP (Rezaei *et al.,* 2005).

This procedure was followed by the incubation with goat anti-mouse IgG (H+L) horseradish peroxidase conjugate (at 1/1000) (Molecular Probes, Germany). All antibody dilutions were done in 1 % non-fat milk in TBS-T. Recombinant PrP bands were detected on an X-ray film X-OMAT Kodak, (USA) after treatment with by enhanced chemiluminescence reagent (ECL) (Pierce, USA). The lowest detectable amount of recPrP by the analysis was ~10 ng per lane.

3. Results

3.1 Effect of earthworm gut environment on microbial community of soil

3.1.1 Preliminary studies of changes of microbial community in the substrate upon passage through the earthworm gut

3.1.1.1 Characterization of the microbial population with FISH

Changes microbial community composition through the passage of the gut of autumn-collected *L. terrestris*

Alphaproteobacteria were a dominant group in the soil and gut samples but their total number became 10-times lower in the cast (Fig. 4A). Relative numbers of *Betaproteobacteria* and *Firmicutes* were high in the soil, shifted down in the gut content, and almost restored in the excrements. *CFB*, *Gammaproteobacteria*, and *Actinobacteria* were augmented during passage although amount of γ-proteobacteria in the gut content was low (Fig. 4A). Yeast extraordinary mounted their numbers in the gut contents, but they were detected neither in the soil, nor in the cast (Fig. 4A). Single cells of *Deltaproteobacteria* were observed in the soil and cast samples. *Archaea* were not detected in any source. Bacteria related to *CFB* group, *Firmicutes*, and β- *and* γ-*proteobacteria* were more numerous in the gut-wall samples in compare with gut content (Fig. 4A). Ratio of bacteria hybridized with to the *Bacteria*-specific probe (EUB338) to those stained with DAPI (EUB338/DAPI) was lower in the soddy-podzolic soil in comparison with excrement (0,20 and 0,29 respectively) and even lower in the gut (gut wall sample 0,19; gut content sample 0,17).

Changes in microbial community composition through the passage of the gut of *A. caliginosa*

Autumn-collected A. caliginosa

Passage through the gut significantly decreased numbers of *Alphaproteobacteria* (Fig. 4B) in contrast to *Betaproteobacteria* who exhibited an opposite behavior. Index of the former grew up significantly in the gut content and was not changed notably in the cast (Fig. 4B). *Gammaproteobacteria* as *Firmicutes* reduced their numbers in the gut content and restored their relative densities in the excrements. Changes of *CFB* and *Actinobacteria* were similar – increasing in the gut content and slight decreasing in the excrements (Fig. 4B). Composition of microbial

community on the gut walls was different from that one of the gut content. Only *Betaproteobacteria* had similar abundances as in the gut contents (Fig. 4B). Numbers of *Actinobacteria*, *CFB,* and *Alphaproteobacteria* were lower in the gut-wall samples. Opposite results were noticed for *Firmicutes* and *Gammaproteobacteria*. The differences were most remarkable for *Firmicutes* (Fig. 4B).

Spring-collected A. caliginosa

We did not evaluate the gut wall microbial community composition in this earthworm group. While *Alphaproteobacteria* gradually increased the numbers in the gut contents and excrements, *CFB* and yeasts increased their densities in the gut but decreased in the cast. Passage through the gut caused strong reduction of *Betaproteobacteria* and *Gammaproteobacteria* already in the gut and in the excrements the indices were the same (Fig 4C). *Firmicutes* declined in the gut content and restored their numbers in the cast. Quantity of *Actinobacteria* and *Archaea* was pretty stable in all samples (Fig. 4C). Separate single cells of *Deltaproteobacteria* were observed in each sample.

Ratio EUB338/DAPI in the autumn- and spring-colleted soddy-podzolic soil was comparable (0,20 and 0,18) and in the cast samples this index was also higher (0,30 and 0,23 autumn- and spring-colleted earthworms respectively), fluctuating in the gut content samples (0,16 and 0,27).

Changes in microbial community composition through the passage of the gut of *E. fetida*

Unlike in the soddy-podzolic soil and in the earthworms populating that substratum, EUB338/DAPI ratio was much higher in the compost and in the cast of earthworm *E. fetida* (0,42 and 0,35 respectively), this ratio was the lowest in the gut content (0,15).

One of the most notable events was the 8-fold increase of numbers of *CFB* bacteria in the gut content in comparison with the compost and a consecutive two-fold reduction of their numbers in the cast. Significant growth of the population of *Actinobacteria* and *Alphaproteobacteria* took place as well (*Alphaproteobacteria* became a dominant group) with a simultaneous strong reduction of *Beta*- and *Gammaproteobacteria* (Fig. 4D). Yeasts and *Archaea* were detected in the compost in quite low concentration (Fig. 4D). Few cells of *Deltaproteobacteria* and 28 fragments of mycelia and just 2 yeast cells were observed in the gut content among approximately 1000 total cells counted in 30 in total microscope fields. Numbers of *Actinobacteria* and *Betaproteobacteria* in the gut-wall samples were twice and three-fold higher than in gut content, respectively. In contrast to that, *Alphaproteobacteria* had two-fold lower proportion on the gut walls. *Gammaproteobacteria*

and *Firmicutes* were not detected in the community of gut wall. The *CFB* had similar abundance in the gut-wall and gut-content samples (Fig. 4D).

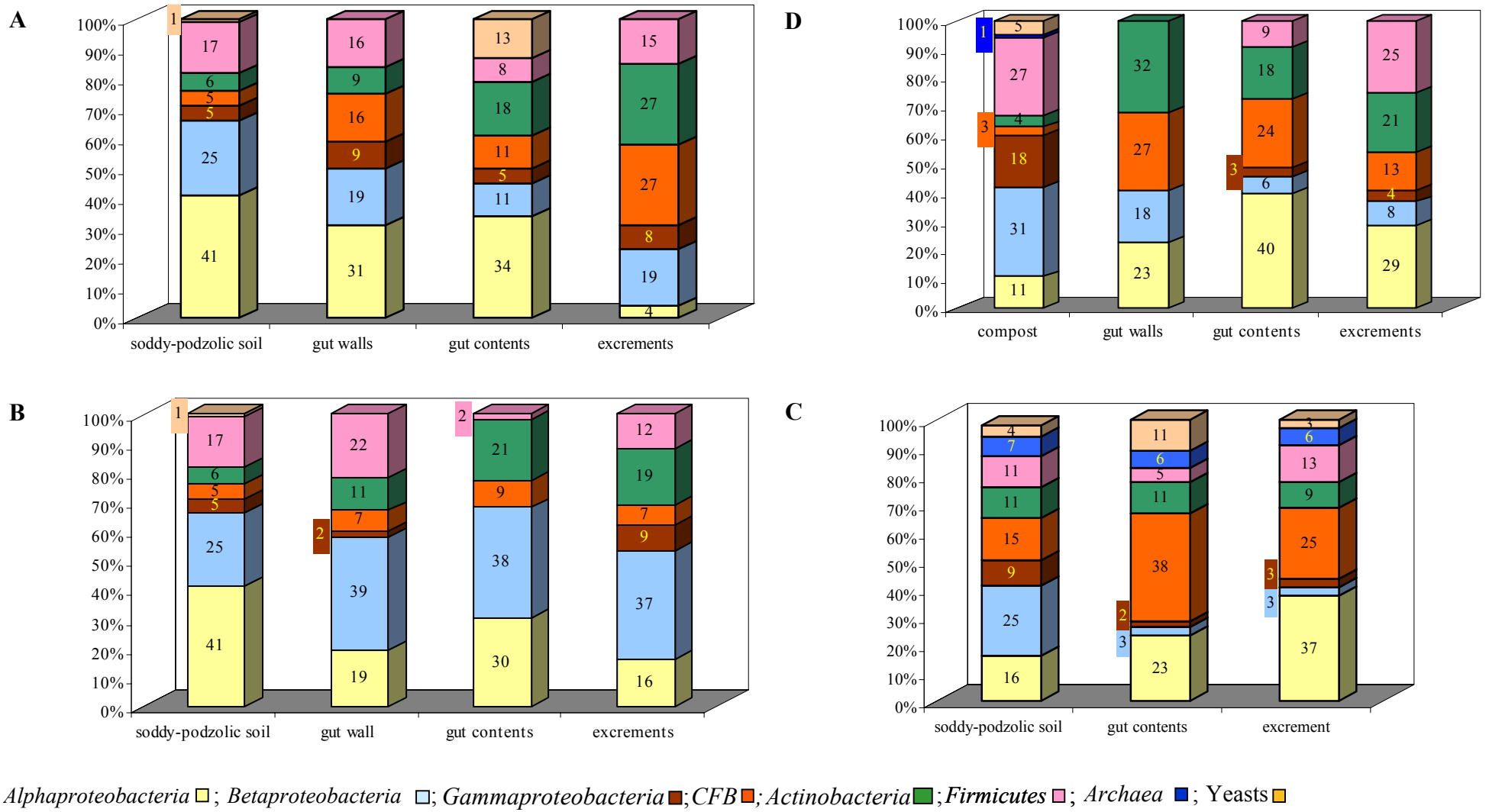


Figure 4. Changes of relative abundance of majortaxonomic groups of microorganisms in substratum passing through the gut. **A:** autumn-collected soddy-podzolic soil and *L. terrestris*; **B:** autumn-collected soddy-podzolic soil and *A. calliginosa*; **C:** spring-collected soddy-podzolic soil and *A. calliginosa*; **D:** horse manure compost and *E. fetida*.

3.1.1.2 Clone libraries

In total, 305 unique bacterial clones were identified in 12 libraries. Except of bacteria we detected the unspecifically amplified 12S mitochondrial and 18S rRNA genes of earthworms and other eukaryotic organisms in gut content and cast libraries.

Proteobacteria had a highest numbers among all bacterial clones (54%). The most numerous phylum was *Gammaproteobacteria*: 83 clones (27%) from 7 orders. *Alphaproteobacteria* (14%) were represented by 7 and *Betaproteobacteria* (11%) by 5 orders. *Deltaproteobacteria* were minor in the clone libraries (5 clones, 2%). *CFB* were the second numerous group of bacteria (61 clones, 20%) represented by *Flavobacteria* (detected mostly in the gut content and cast), *Sphingobacteria* (detected mostly in soils and compost) classes. Among *Actinobacteria* (25 clones, 8%) clones from *Micrococcineae* suborder were the most frequent. *Firmicutes* (34 clones, 8%) were detected in each library, but class *Mollicutes* was found only in gut and cast of earthworms; class *Bacilli* (clones linked to *Bacillus*, *Paenibacillus*, *Brevibacillus*, *Aeurinibacillus*, *Geobacillus* and *Anoxybacillus* genera) was the common in the majority of libraries except of gut wall library of *L. terrestris*; *Clostridia* (*Ruminococcus* genus) were the only class of *Firmicutes* identified in horse manure compost and autumn-collected *A. cliginosa* cast library (related to *Desulfotomaculum* genus).

Acidobacteria (*Fibrobacteres/Acidobacteria* group), *Myxococcales*, and *Cloroflexi* were found only in gut content or cast libraries. *Verrucomicrobia* and *Planctomycetes* were found in all sources but they were more abundant and identical to each other in the gut content and cast libraries (Suppl. Fig. 34).

Clones of some bacterial taxa clearly affiliated their taxonomic placement distanced from known species. The clusters were formed by clones equally distant from *Succinivibrionaceae* and *Legionellaceae* (*Gammaproteobacteria*) in compost, gut content of *E. fetida* and cast of *L. terrestris.* Other bacterial clones were considered as “unclassified” and apparently belonged to *Acidimicrobidae* and *Actinobacteridae* subclasses (*Actinobacteria*) with unclear affiliation (Suppl. Fig. 37, 39). Some clones from *Sphingobacteriales* family also formed a cluster together with a symbiont sf. *Flavobacterium* of *Tetraponera binghami* (AF459795) as closest hit (van Borm *et al*., 2002).

Some identical OTUs were detected in the substrata (compost or soil) and in gut libraries of earthworm inhabiting these substrata, or in the gut and cast libraries, but none were found in all three samples, substratum, gut, and cast.

Autumn-collected soddy-podzolic soil

Proteobacteria were dominated class of microorganisms making up to 78 % among 28 clones. Among *Gammaproteobacteria* (13 OTUs) a half of the clones was related to bacteria of family *Xantomonadaceae*; other numerous clones were from family *Pseudomonadaceae* (including genus *Pseudomonas*) (Suppl. Fig. 37). *Alphaproteobacteria* (6 OTUs) were represented by organisms from orders *Rhizobiales*, *Sphingomonadales*, and *Rhodospirillales* (Suppl. Fig. 35). *Betaproteobacteria* (3 OTUs) were corresponding to families *Alcaligenaceae* and *Burkholderiaceae*, and bacteria of *CFB* (3 OTUs) related to classes *Flavobacteria* and *Sphingobacteria* (Suppl. Fig. 36, 38). *Verrucomicrobia*, *Planctomycetes*, and *Firmicutes* (class *Bacilli*) were present in the library only as single clones (Suppl. Fig. 34, 39).

Autumn-collected L. terrestris

Microbial community composition in this variant was more similar between the gut content and cast in contrast to soil and gut-wall libraries. But affiliation of OTUs was stronger between gut-content and cast libraries. The same families as in the soddy-podzolic soil but without *Sphingomonadales* were represented by *Alphaproteobacteria* (6 OTUs) in the gut-wall library. *Betaproteobacteria* were detected in each library by single clones related to family *Commamonadaceae* (3 OTUs) (Suppl. Fig. 36). *Gammaproteobacteria* were represented by clones from families *Aeromonadaceae* (3 OTUs) and *Schevanellaceae* (1 OTU) in the gut wall, and by families *Legionellaceae* (1 OTU) and *Pseudomonadaceae* (2 OTUs) in another libraries. Five pairs of OTUs from *Flavobacterium* genus (*CFB*), 4 pairs of OTU from *Planctomycetes* and 1 pair of OTU from *Verrucomicrobia* were represented in the gut content and cast libraries (Suppl. Fig. 34, 38). *Actinobacteria* were detected in a single OTU (*Microbacterium* genus) in the gut-wall and two matching OTU (*Nocardioides* genus) in the gut content and cast; another clone of *Mycobacterium* genus was discovered in the cast library (Suppl. Fig. 39). Three clones of *Bacilli* (class *Firmicutes*) linked to the genus *Bacillus* were found in the gut content and cast libraries.

We also detected a single OTU (2 clones) closely related to roundworms of genus *Rhabditis* (*Nematoda*) (Fig. 5) (18S rDNA sequence identity 98%). Eukaryotes from family *Monocystidae* (*Apicomplexa*; 1 OTU, 3 clones) were also present in the library; sequence identity of 18S rDNA to closest hit (*Monocystis agilis* AF213515) was 90%.

Figure 5. Roundworms of genus *Rhabditis* (*Nematoda*) found alive in the soddy-podtolic soil and the cast of *L. terrestris*: magnification, ×8; bar, 1cm.

Autumn-collected A. caliginosa

The clone libraries from *A. caliginosa*-derived environments did not show a clear consistency between the libraries in contrast to *L. terrestris*. *CFB* were more abundant in the gut content library. Numbers of OTUs from orders *Flavobacteriales* and *Sphingomonadales* were comparable (6 and 5 respectively) (Suppl. Fig. 38). *Firmicutes* were also more frequent in the gut content library (4 OTUs from class *Bacilli*), than in soil or cast (1 OTU from class *Clostridia*) (Suppl. Fig. 39). In contrast to *CFB* and *Firmicutes*, compositions of *Alpha-*, *Beta-*, and *Gammaproteobacteria* were more inconsistent in the cast (4, 3, and 4 different taxa in each subclass respectively) and soil libraries than in the gut content (1, 1, and 1 taxon from each subclass respectively) (Suppl. Fig. 35-37). *Actinobacteria* of suborders *Micrococcineae* (6 OTUs) and *Propionibacterineae* (1 OTU) were detected only in the cast library (Suppl. Fig. 39) A single clone represented *Verrucomicrobia* and *Chloroflexi* (class *Thermomicrobia*) in the cast library (Suppl. Fig. 34).

Spring-collected soddy-podzolic soil and A. caliginosa

The clones derived from *CFB* were abundant in the soil and gut-content libraries and scarce in the cast library. All clones in soil library were matching the bacteria from order *Sphingomonadales*; most of the clones had a high affinity to symbiont sf. *Flavobacterium* of *Tetraponera binghami* (AF459795). There were 3 OTUs in the gut content library similar to those from the libraries of autumn-collected *A. caliginosa* and related to genera *Flavobacterium* and one OTU to *Flexibacter*. Bacteria of *CFB* in the cast were detected in single OTU close to symbiont sf. *Flavobacterium* of *Tetraponera binghami* (AF459795) (Suppl. Fig 38). *Proteobacteria* were not very numerous in the soil library and represented by 2 OTUs from *Alphaproteobacteria*, 3 OTUs from

Betaproteobacteria, and single OTU from *Gammaproteobacteria* (Suppl. Fig. 35-37). This class of bacteria was more regularly present in cast than in gut content: 3 and 7 OTUs belonged to *Alphaproteobacteria* were found, correspondingly. *Betaproteobacteria* (3 OTUs) were detected only in the cast library. *Gammaproteobacteria* were also seldom in both, gut content library (2 OTUs) and in the cast library (3 OTUs). One OTU from order of *Desulfomonadales* and 2 OTUs from orders of *Desulfomonadales* and *Myxococcales* (*Deltaproteobacteria*) were recognized in the gut content and cast libraries respectively (Suppl. Fig. 34). *Firmicutes* from genera *Geobacillus* and *Brevibacillus* (3 OTUs, class *Bacilli*) and genus *Desulfotomaculum* (2 OTUs, class *Clostridia*) occured in the soil library. 2 OTUs of genus *Geobacillus*, class *Bacilli* was found in the cast library. No *Firmicutes* were detected in the gut content library. *Actinobacteria* were lacking in the soil but present in the cast (3 OTUs) and frequent in the gut content libraries (7 OTUs) (Suppl. Fig. 39).

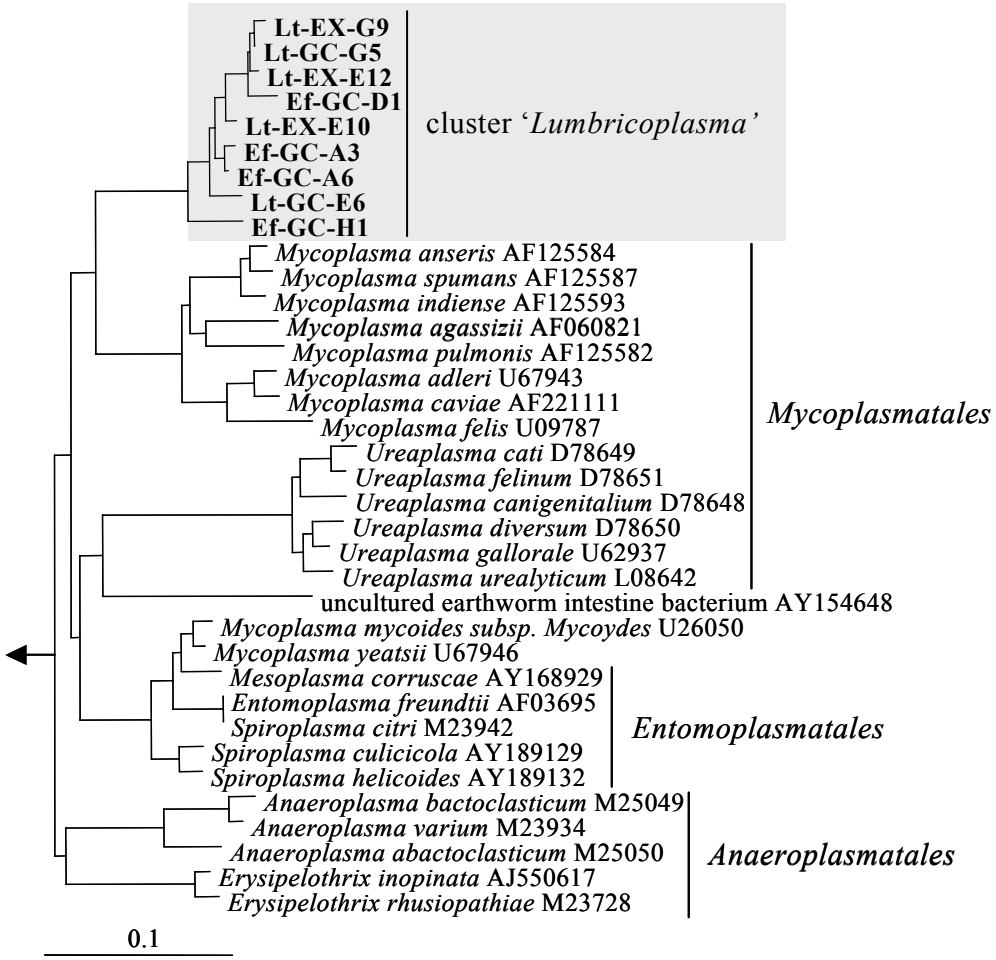
Horse manure compost and E. fetida

Proteobacteria were the most numerous group in the gut content and cast libraries. *Alphaproteobacteria* were detected only in compost library (3 OTUs). Two OTUs represented *Betaproteobacteria* in the compost library and 1 OTU in the cast library. *Gammaproteobacteria* were the most numerous class with highest diversity in the compost library (8 OTUs from 5 families including *Legionellaceae*). Diversity was lower in the gut content (6 OTUs from families *Legionellaceae* and *Pseudomonadaceae*), and lowest in the cast library (2 OTUs from *Aeromonadaceae* and *Enterobacteriaceae*) (Suppl. Fig. 37). Single OTU related to the genus *Geobacter* (*Deltaproteobacteria*) was detected in the compost library. (Suppl. Fig. 34). *CFB* of classes *Sphingobacteria* and *Flavobacteria* (8 and 3 OTUs respectively) were enriched in the compost library. Bacteria from the class *Sphingobacteria* were detected in both libraries (2 OTUs in gut content and 1 OTU in the cast). Bacteria linked to the genus *Flavobacteria* appeared only in the cast (2 OTUs) (Suppl. Fig. 38). Single OTU of *Planctomycetes* and *Actinobacteria* (from unclassified cluster) were detected in the compost library (Suppl. Fig. 34, 39). Single OTU from *Actinobacteria* recognized in the cast library was related to *Acidomicrobium ferrooxidans* (subclass *Acidimicrobidae*). *Firmicutes* in the compost library were affiliated to genus *Ruminococcus* of class *Clostridia* (4 OTUs). All *Firmicutes* detected in the gut content library were related to *Mollicutes* (4 OTUs) (data not shown), and the single OTU found in cast library was close to *Bacillus sphaericus* (Suppl. Fig. 39).

Apart from bacteria, we amplified 18S rDNA from eucarya of family *Chlorophyceae* (1 OTU) in the cast library (closest hit was *Chlamydomonas pitschmannii* (U70789); sequence identity <90%).

3.1.2 Bacteria of class *Mollicutes* in the earthworm tissues

Apart from other microbes, bacteria belonged to class *Mollicutes* (*Firmicutes*) were detected in the clone libraries of cast and gut content of *E. fetida* and *L. terrestris*. The clones were abundant in the libraries, affiliated together in the cluster, and had low sequence similarity (83%) to the closest hit (*Mycoplasma pulmonis* AF125582) (Fig. 6); the clones from the same origin had varied sequence identity each to other (92-99%). *Mollicutes*-like organisms (MLO) have been reported to be present by a single clone in the intestine library of *L. rubellus* (Singleton *et al.,* 2003) but its sequence identity both to our clones was low (best hit ~80%) as well as to other described *Mollicutes*. Significant evolutionary distance of novel discovered bacterial clones from the other bacteria of the class *Mollicutes*, source of isolation (earthworms of family *Lumbricidae*), and well-known host specificity of *Mollicutes* make the bacteria from novel discovered cluster to be thought a new



taxonomy group, named cluster '*Lumbricoplasma'* candidates.

Figure 6. Phylogenetic distribution of the clones (~ 1000 bp length) from class *Mollicutes* in the samples of gut content (GC) and casts (EX) of two earthworm species: *L. terrestris* (Lt); *E. fetida* (Ef).

On the baseis of sequence analysis of '*Lumbricoplasma'* cluster, the '*Lumbricoplasma'*-specific 5'-labelled AlexaFluor546 nucleotide probe LUM1225 was synthesized by Invitrogen (Germany). The

localization of the bacteria from cluster '*Lumbricoplasma'* was studied using FISH analysis. The microscopic analysis has turned out the nodules localization of the rod-shaped bacteria mostly in the ring and longitudinal muscles and coelom tissues but also in the gut tissues and outer epidermis (Fig. 7-10).

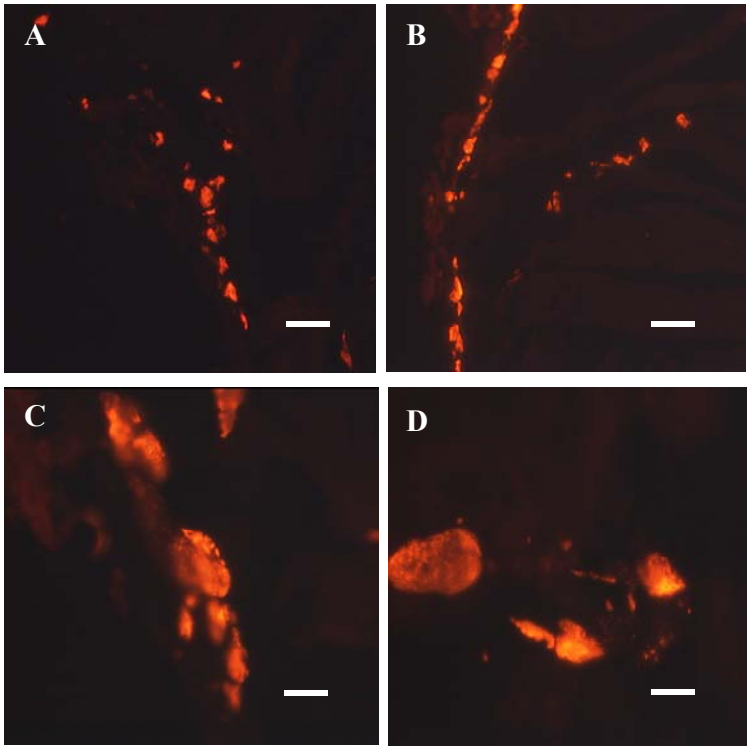


Figure 7. Bacteria of cluster '*Lumbricoplasma*' and their tissue specimen in the ring and longitudinal muscles of the earthworm *L. terrestris* visualized with '*Lumbricoplasma*'-specific nucleotide probe using fluorescent microscopy. **A, B**: magnification, ×200; bar, 120 µm. **C, D**: magnification, ×630; bar, 20 µm.

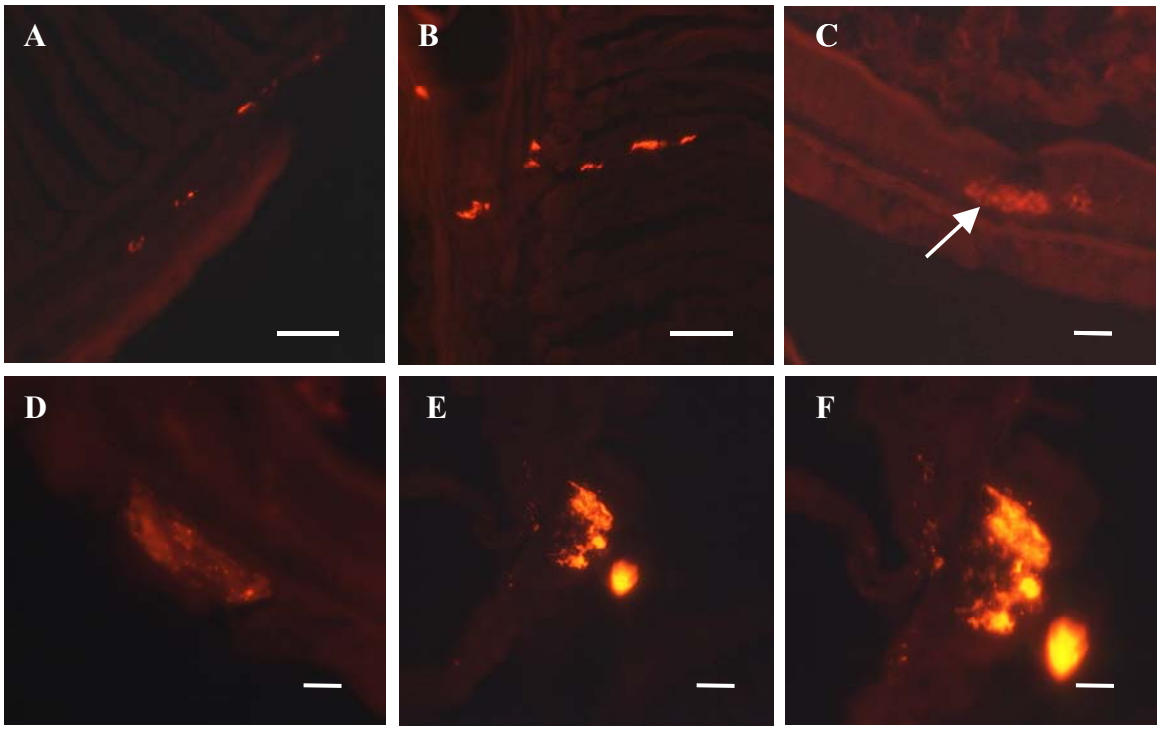


Figure 8. Bacteria of cluster '*Lumbricoplasma*' and their tissue specimen in the earthworm *A. caliginosa* visualized with '*Lumbricoplasma*'-specific nucleotide analysis using fluorescent microscopy. Ring muscles (**A**): magnification, ×200; bar, 150 µm. Longitudinal muscles (**B**): magnification, ×200; bar, 150 µm. Different parts of gut wall: (**C**) magnification, ×400; bar, 40 µm; (**D**) magnification, ×400; bar, 40 µm; in the same field (**E**) magnification, ×400; bar, 40 µm; magnification, ×630; bar, 20 µm (**F**);

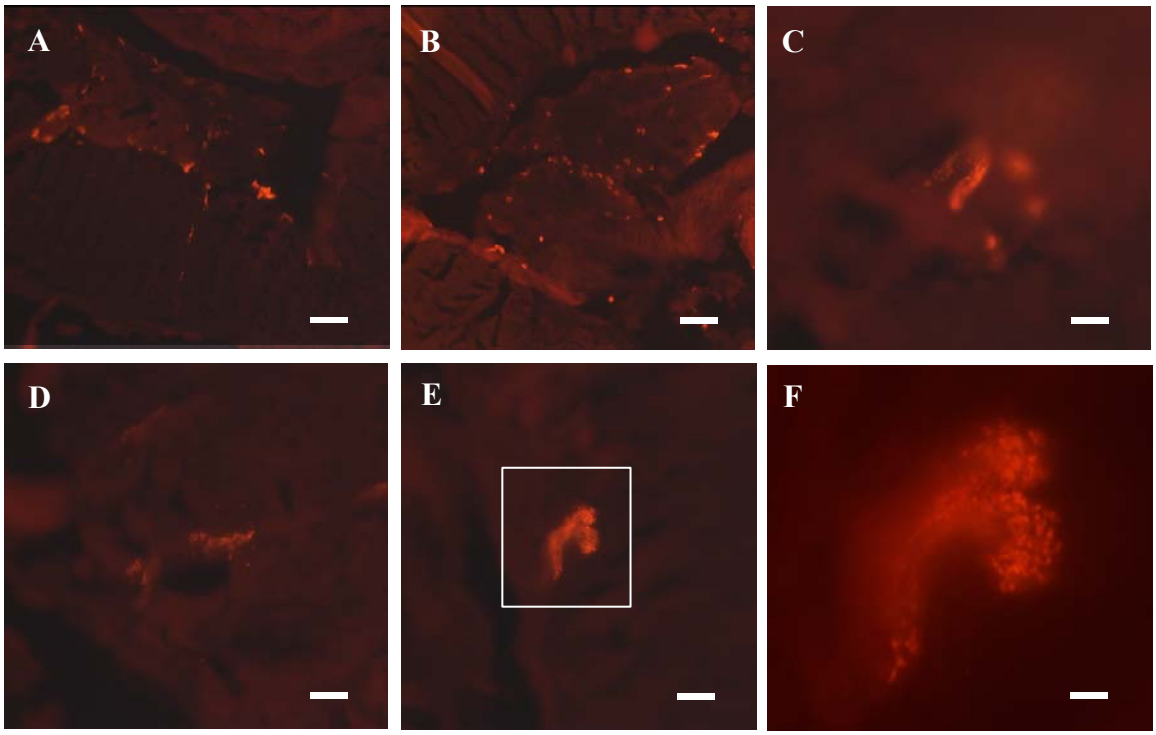


Figure 9. Bacteria of cluster '*Lumbricoplasma*' and their tissue specimen in the earthworm *E. fetida* visualized with '*Lumbricoplasma*'-specific nucleotide probe analysis using fluorescent microscopy. Coelom (**A, B**): magnification, ×200; bar, 60 µm. Gut wall (**C**): magnification, ×400; bar, 30 µm;

Longitudinal muscles: magnification, ×400; bar, 30 µm (**D**); magnification, ×400; bar, 30 µm (**D**), in the same field magnification, ×630; bar, 10 µm (**E**).

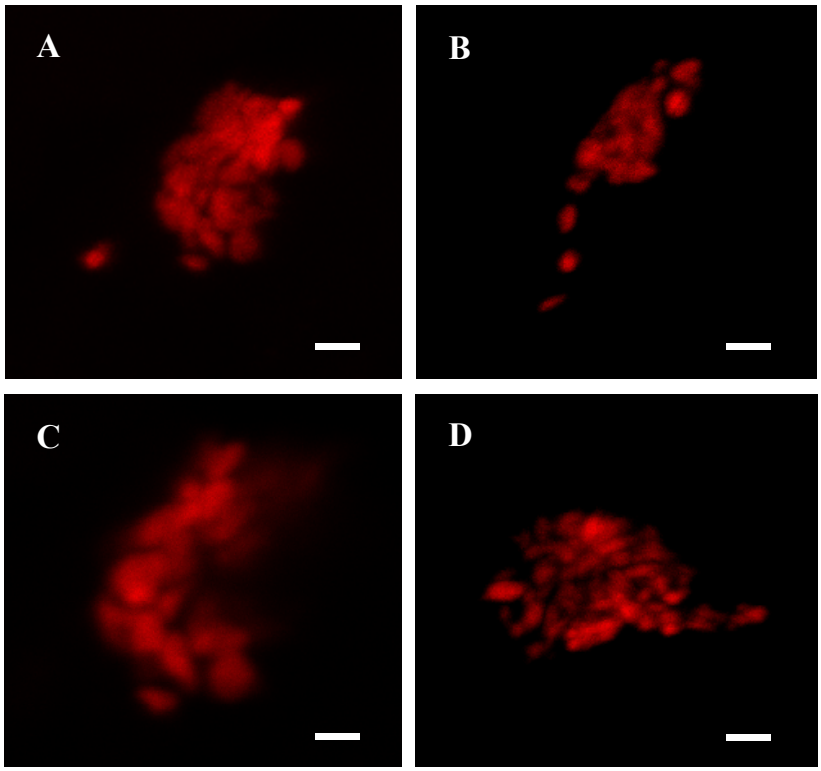


Figure 10. Bacteria of cluster '*Lumbricoplasma*' visualized with '*Lumbricoplasma*'-specific nucleotide probe in the longitudinal muscles of *E. fetida* using laser confocal microscopy. **A-D**: magnification, ×1000; bar, 1 µm.

3.1.3 FISH and SSCP analysis of microbial communities in the substratum used for prion proteolytic assay

For recPrP proteolysis assay the samples of soddy-podzolic soil and earthworm casts collected in the spring 2005 were used. Initially they were examined with FISH and SSCP analyses to reveal the microbial community composition in these particular samples.
Preliminary studies of microbial communities with FISH revealed gradually changes of microbiota upon gut passage; this is why only initial substratum and cast (and not the gut content) were chosen for the forthcoming analysis.

3.1.3.1 Characterization of the microbial population with FISH

Bacterial population changes upon passage through the gut of L. terrestris
Alpha-, *Betaproteobacteria*, and *CFB* bacteria were dominant in the bacterial soil population and had comparable number each to other. The rest bacterial groups comprised in total 11%. Bacteria of *CFB* group upon passage increased their relative numbers almost twice and reached 50% mark. Proportion of *Alpha*-, *Gammaproteobacteria*, and *LGC* bacteria decreased two-fold (14, 2, 1% respectively), whlie *Betaproteobacteria* and *Actinobacteria* kept the same or almost the same numbers (*Actinobacteria* had 3 and 4% in soil and cast respectively) (Fig. 11).

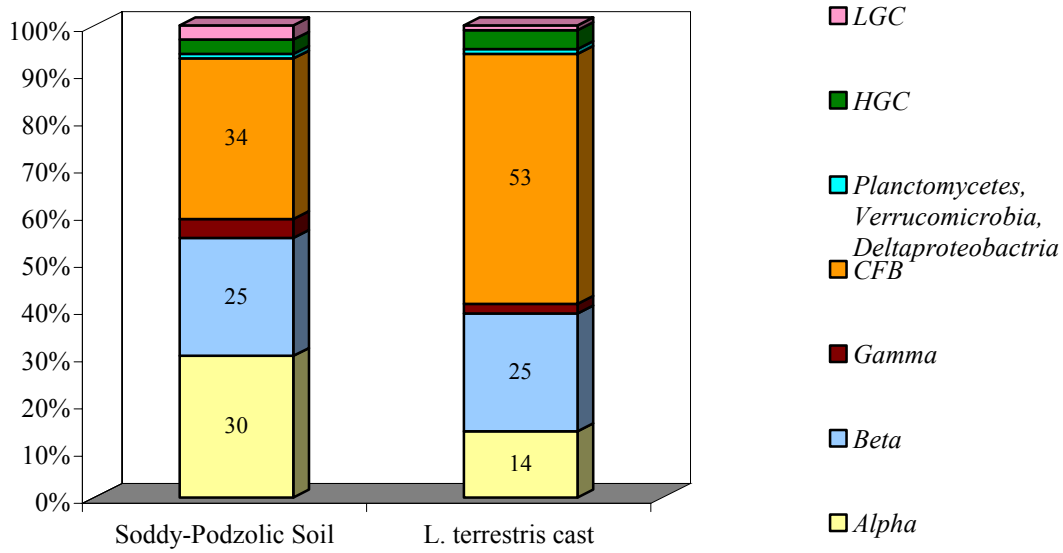


Figure 11. Composition of different phylogenetic groups of microorganisms in the soddy-podzolic soil and cast *L. terrestris.*

Numbers of *Deltaproteobacteria*, *Verrucomicrobia* and *Planctomycetes* were very low (less than 1%) and did not change notably upon passage through the earthworm gut (Fig. 11).

Ratio of EUB338/DAPI in the soddy-podzolic soil was similar (0,21) to that determined during the previous studies, this value was higher in the cast samples of *L. terrestris* and *A. caliginosa* (0,27 and 0,26 respectively).

Bacterial population changes upon passage through the gut of A. caliginosa

Bacterial population alterations upon passage through the gut of *A. caliginosa* were similar to those *L. terrestris* (Fig. 12). Bacteria of *CFB* group also became dominant in the cast, *Alpha*-, *Gammaproteobacteria*, and *Firmicutes* (LGC) diminished their numbers. *Actinobacteria* (HGC) and *Planctomycetes* retained same relative abundances and *Betaproteobacteria* slightly increased their density (Fig. 12). Similarly to the cast of *L. terrestris*, *Verrucomicrobia*, *Planctomycetes* and *Deltaproteobacteria* were not abundant in the cast of *A. caliginosa*.

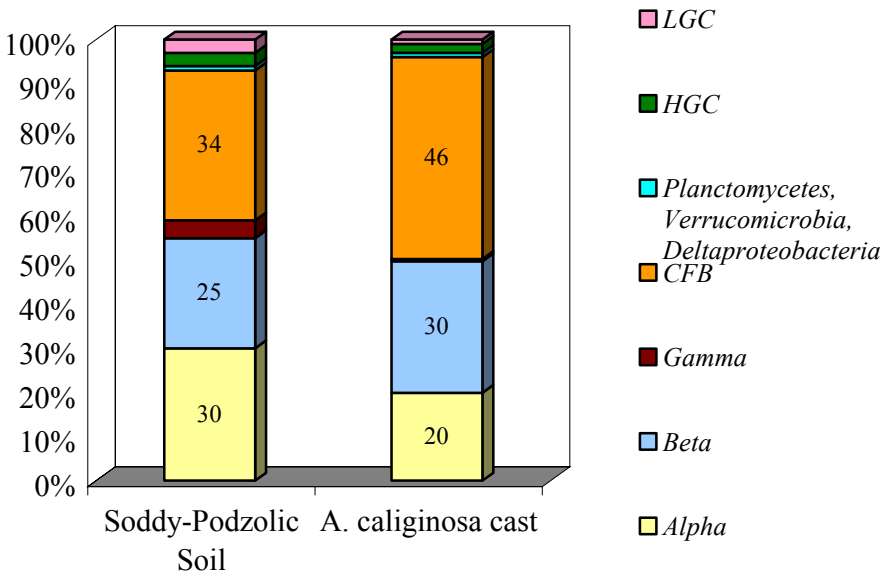


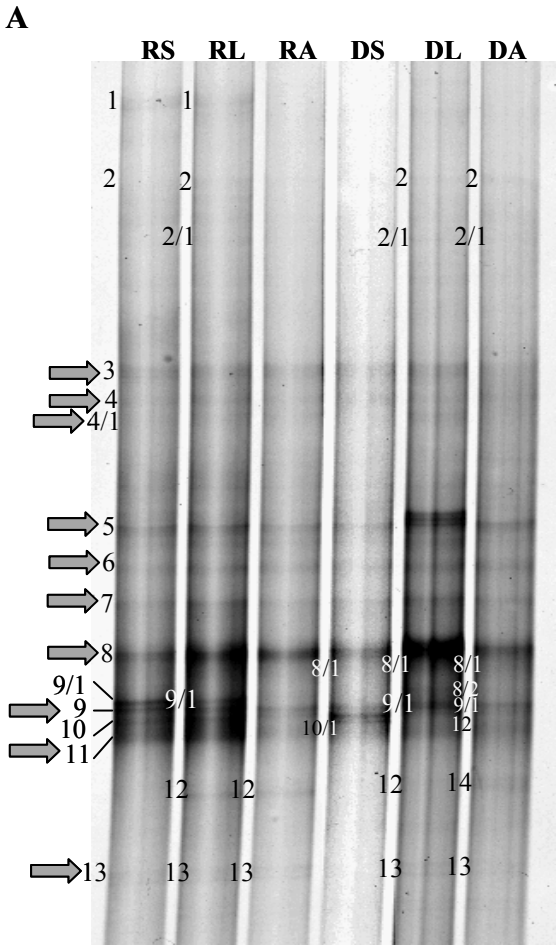
Figure 12. Composition of different phylogenetic groups of microorganisms in the soddy-podzolic soil and cast *A. caliginosa*.

Bacteria of the phyla *Verrucomicrobia*, *Planctomycetes* and *Deltaproteobacteria* were the minor members of bacterial communities in the soil and earthworm casts, since their proportion hybridized with specific probes was about 1%.

3.1.2.2 SSCP analysis

SSCP analysis with the nonspecific primer set

SSCP profiles of the samples generated from RNA and DNA pool were similar for all samples (Fig. 13A). Many bands (3-9, 12, 13) were common for all samples. Phylogenetic analysis of the sequences from separate bands revealed microorganisms to belong to the following taxonomic groups: α-, β-, γ-, δ-*Proteobacteria*, *CFB*, *Actinobacteria*, *Acidobacteria*, and *Gemmatimonadetes*. Some of the bands running at the same position on the gel consisted of DNA from different resources. The bacteria of *CFB* phyla (particularly from genus *Flavobacteria*) were the most numerous and diverse on the species- and strain-taxonomy level (Fig. 13B). Band S-RL-9a from the casts of *L. terrestris* did not exhibit a strong affiliation to any bacterial taxonomic group and was but related to some uncultured bacterium (AY274120). Among the bacterial amplicons we detected 12S rRNA genes from two different eucaryiotic mitochondrions (S-RL-1a, b) related to the plant-pathogenic fungus *Phytophthora infestans* in the casts samples of *L. terrestris* (Fig. 13B).



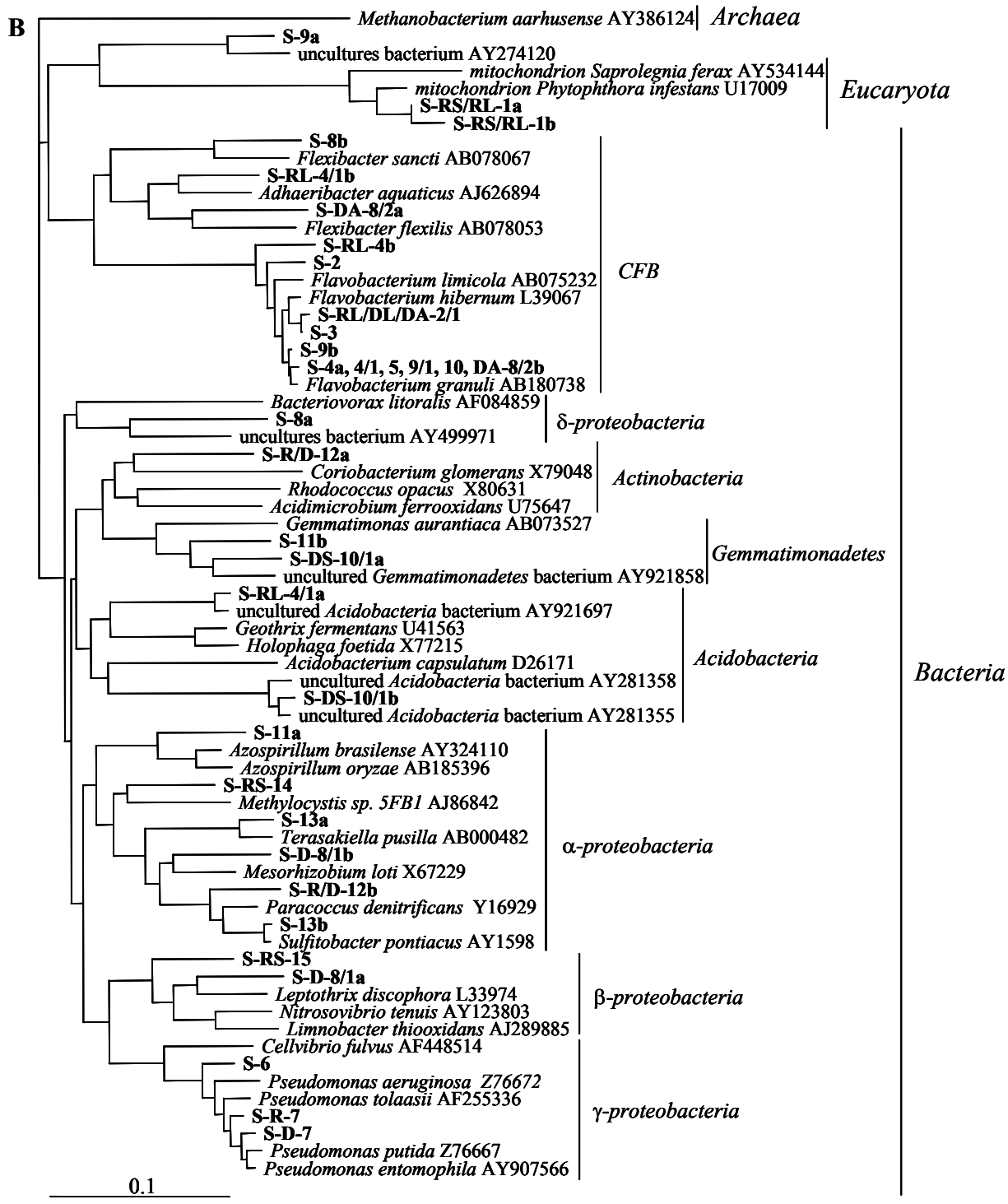
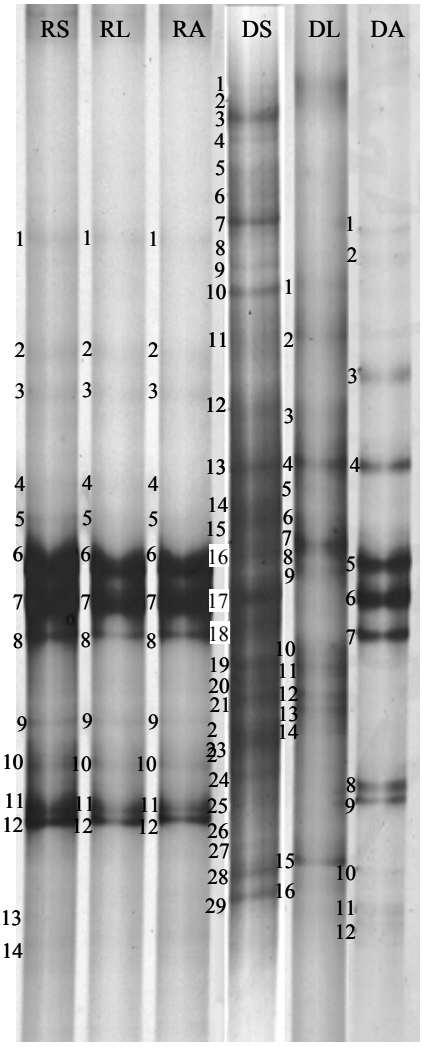


Figure 13. A: SSCP analysis of microbial community with universal SSCP primers. The PCR products were generated from the total RNA (R) and DNA (D). Lanes: soil (S); earthworm *L. terrestris* (L); *A. caliginosa* (A); **B**: Phylogenetic analysis of the bands performed with the unspecific SSCP analysis. Several ribotypes detected in one band represented on phylogenetic tree with number and small letters (a and b). Sequences of the bands running at the same level with identity >98,5% were recognized as the single OTU and are shown on the phylogenetic tree as a single ID.

Alphaproteobacteria-specific assay

SSCP analysis has demonstrated a high number of the bands in the samples and the most numerous were the soil patterns generated from both DNA and RNA pools (Fig. 14A). The phylogenetic analysis of the bands generated from the RNA revealed the bacteria belonged solely to the unclassified *Sphingomonadaceae*, which were very diverse on the species-, and subspecies-taxonomy level. Although some bands were differently running on the gel, they exhibited more similarity between each other, than those running at the same positions even in the neighboring lanes (Fig. 14B). Bands amplified from the DNA belonged to the microorganisms from orders (unclassified *Sphingomonadaceae*, *Sphingomonadaceae*, *Caulobacteraceae*, and *Methylobacteraceae*); the sequence A-DA-2 detected in the *A. caliginosa* cast sample was uncertainly affiliated with the orders *Bradyrhizobiaceae* or *Rhizobiaceae*. Among the bands generated from DNA pool a single band A-DL-6 extracted from *L. terrestris* casts war derived from someorganism from the order *Myxococcales* (class *Deltaproteobacteria)*.

A



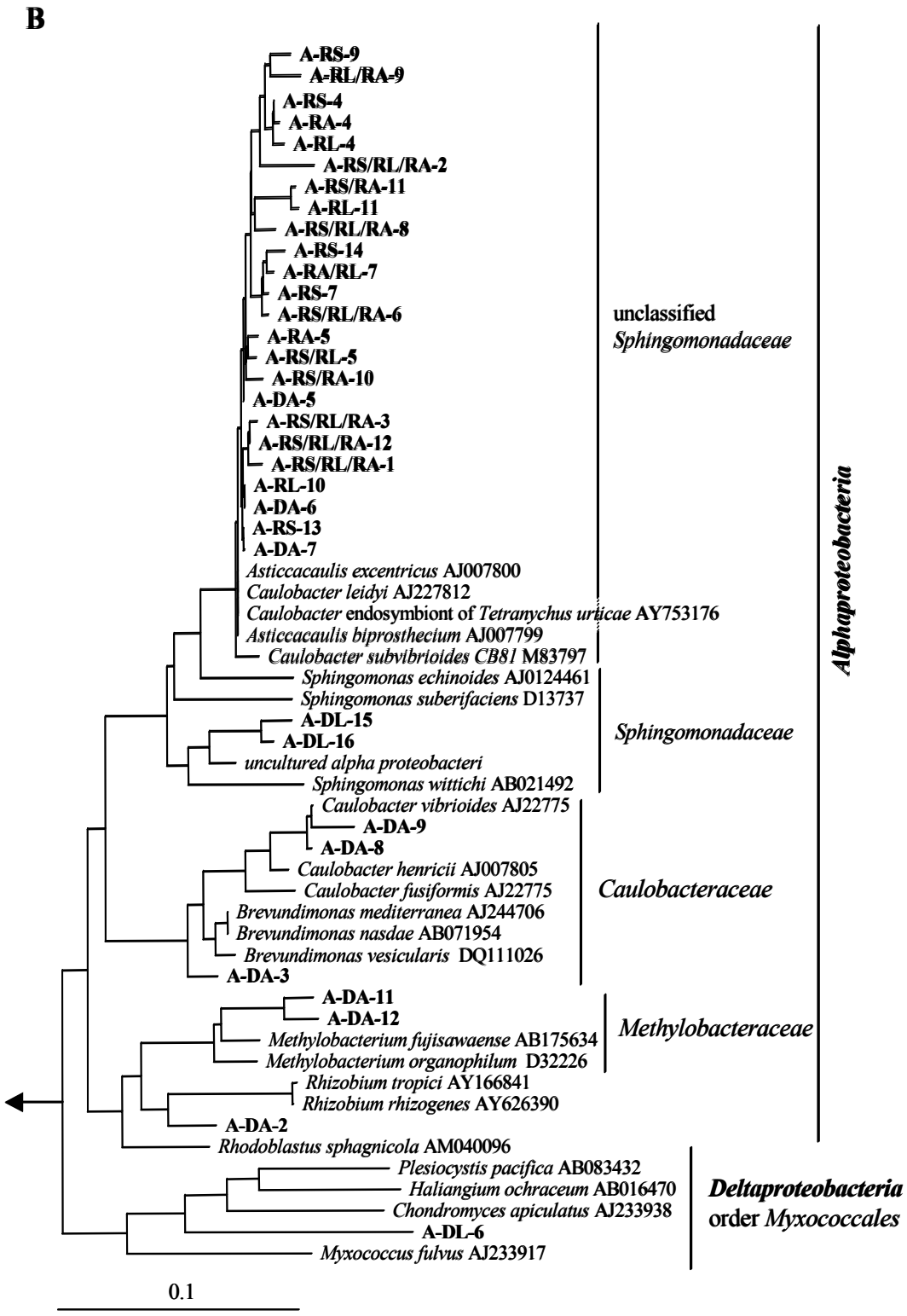


Figure 14. A: SSCP analysis of *Alphaproteobacteria*-specific PCR products generated from total RNA (using reverse transcription) (R) and total DNA (D). Lanes: soil (S); earthworm *L. terrestris* (L); *A. caliginosa* (A); **B**: Phylogenetic analysis of the sequences from the *Alphaproteobacteria*-specific SSCP analysis. Several ribotypes detected in one band represented on phylogenetic tree with the number and small letters (a and b). Sequences with identity >98,5% were recognized as a single OTU and are shown as one ID. .

Betaproteobacteria-specific assay

This class of *Proteobacteria* was represented by a single band in the soil as in the earthworm cast samples generated from RNA (Fig. 15A). The sequences of those bands were similar each to other and could be attributed to the same OTU closely related to *Alcaligenes faecalis* AF155157 (99% of sequence identity). Samples generated from DNA showed numerous bands (13 in the soil and 9 in each cast sample) and similar sequence composition. The sequences were related to bacteria of families *Comamonadaceae*, *Nitrosomonadaceae*, *Oxalobacteraceae*, *Burkholderiaceae*, *Alcaligenaceae*, *Rhodocyclaceae*, and unclassified *Burkholderiales.* The sequences in the samples generated from the DNA and related to A. *fecalis* had >99% of sequence similarity to those generated from RNA (data not shown). Sequences from the top part of the gel (numbers 1-4, 4/1, 5/1) formed a cluster not strongly affiliated to any known bacterial name but apparently belonged to the class *Betaproteobacteria* (Fig. 15B).

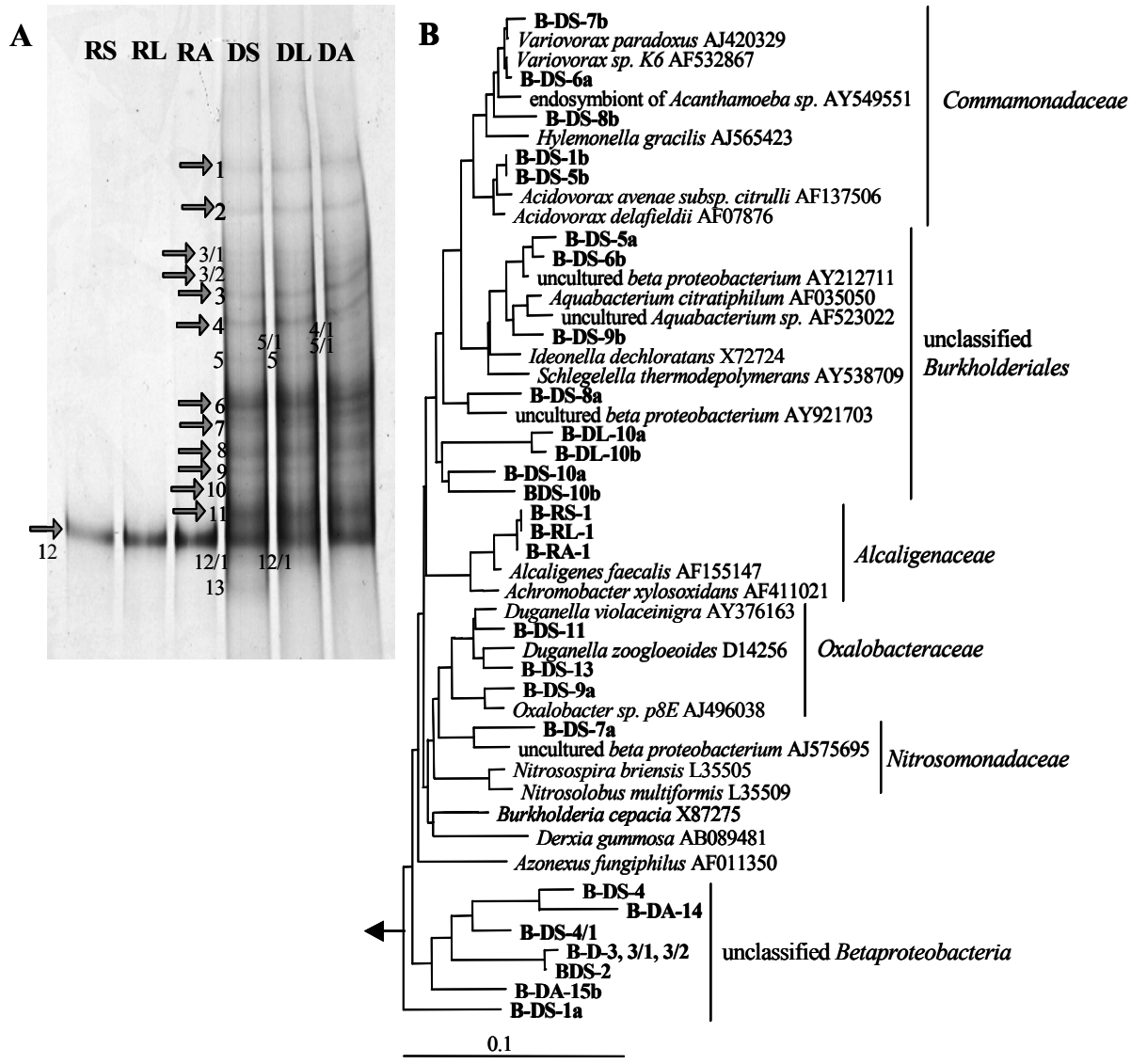


Figure 15. A: SSCP analysis of *Betaproteobacteria* -specific PCR products generated from total RNA (using reverse transcription) (R) and total DNA (D). Bands represented in all profiles marked with arrows. Lanes: soil (S); earthworm *L. terrestris* (L); *A. caliginosa* (A); **B**: Phylogenetic analysis of the phylotypes from the *Betaproteobacteria*-specific SSCP analysis. Several ribotypes detected in one band represented on phylogenetic tree with the number and small letters (a and b). Sequences with identity >98,5% were recognized as a single OTU and are shown as one ID. .

Gammaproteobacteria-specific SSCP assay

PCR product from total DNA with primers specific for *Gammaproteobacteria* was obtained only from soil sample and the fingerprint looked similar to that from generated from RNA, but the latter yielded a higher number of bands. *Gammaproteobacteria* appeared more diverse in the soil in comparison with the samples from the cast of both earthworm species, which were very similar one to another (Fig. 16A).

Sequences of *Gammaproteobacteria* were distributed between 6 clusters related to families *Pseudomonadaceae* (genera *Pseudomonas* and *Cellvibrio*), *Legionellaceae*, *Chromatiaceae* (linked to genus *Nitrosococcus*), *Xanthomonadaceae*, the unclassified bacteria, which were not strongly affiliated to any described bacterial genus but clearly clustered within the class *Gammaproteobacteria*, and unclassified bacteria that are not strongly affiliated neither to classes *Gamma*-, nor to *Betaproteobacteria* (detected in the *A. caliginosa* cast samples band G-RA-34). Three groups of bacteria (genus *Cellvibrio*, family *Chromatiaceae*, and unclassified *Gammaproteobacteria*) were detected only in the soil sample but never in the casts. Bacteria linked to genus *Legionella* were more diverse in the soil samples; the only ribotype detected in the cast of *A. caliginosa* appeared to have a low sequence similarity (<85%) to both described species and sequences found in the soil sample. Composition of *Pseudomonas*-related bacteria was different in the soil samples amplified directly from total DNA (bands 5, 6/1, 6/2 – three OTUs) in comparison to those generated from total RNA by using reverse transcription (bands G-RS-18, 21 (the same OTU) and G-RS-20/1) (Fig. 16B). Most of the recognized ribotypes in the cast samples were linked to *P. putida* and *P. entomophila*. Among them two bands (G-RL-30 and G-RA-37) in the casts of each earthworm species belonged to the same OTU, while the rest of the bands (4 in each cast sample) were associated with another single OTU (Fig. 16B). Passage through the earthworm gut caused decreasing diversity of *Gammaproteobacteria* and increasing detectable number of species- and subspecies-level variants of bacteria closely related to *P. putida* and *P. entomophila* (Fig. 16B). The band G-RA-33 was derived from family *Xanthomonadaceae*, similarly to G-RS-12 related to genus *Stenotrophomonas* generated from RNA of the soil sample.

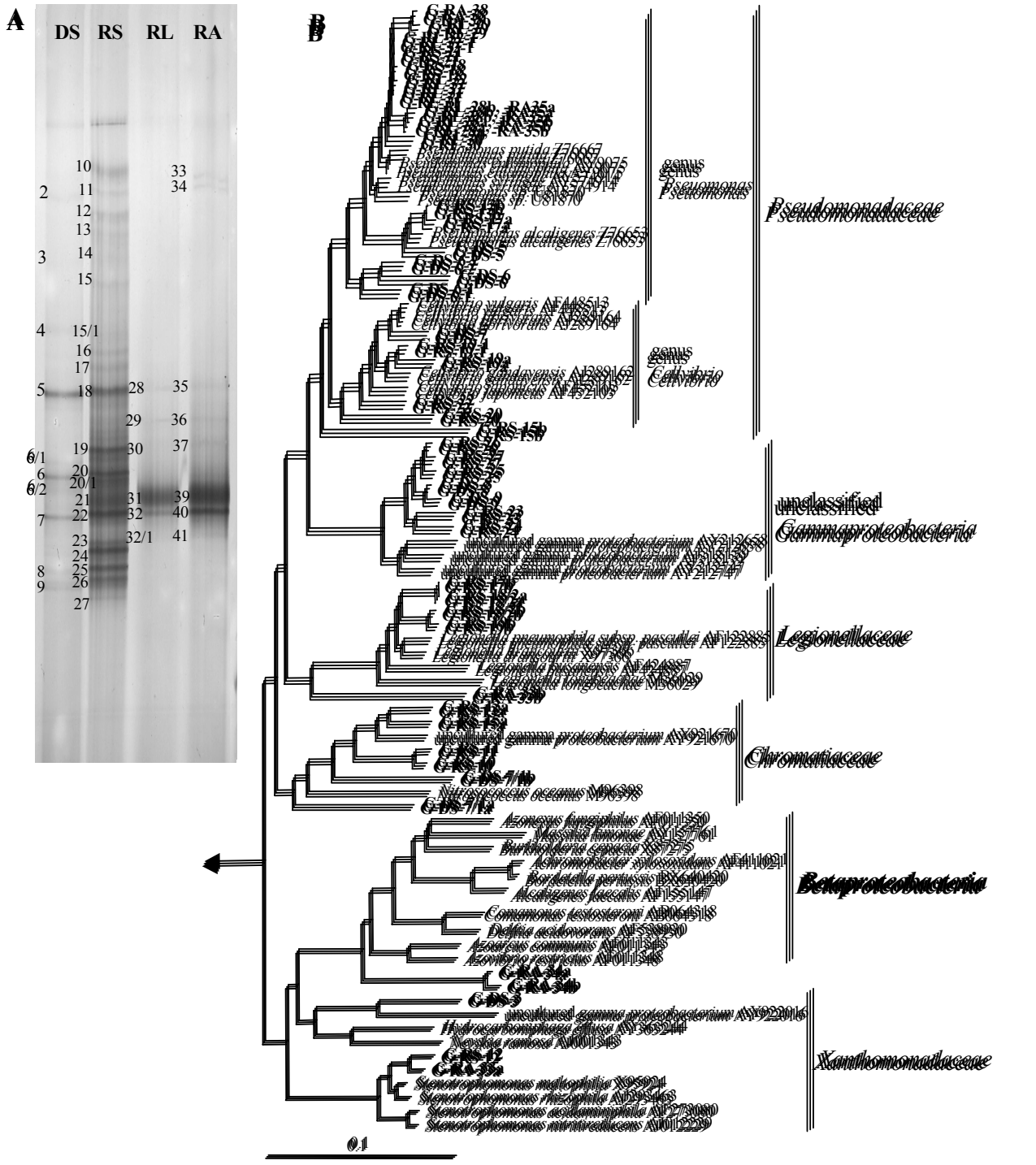


Figure 16. A: SSCP analysis of *Gammaproteobacteria*-specific PCR products generated from total RNA (using reverse transcription) (R) and total DNA (D). Lanes: soil (S); earthworm *L. terrestris* (L); *A. caliginosa* (A); **B**: Phylogenetic analysis of the sequences from the *Alphaproteobacteria*-specific SSCP analysis. Several ribotypes detected in one band represented on phylogenetic tree with the number and small letters (a and b). Sequences with identity >98,5% were recognized as a single OTU and are shown as one ID.

CFB-specific SSCP assay

Amplifications with primers specific for *Cytophaga-Flavobacteria* group of *Bacteroidetes* were successful from both total dsDNA and ssDNA (generated from total RNA). SSCP profiles did not appear to be significantly different among the soil and earthworm cast patterns generated from DNA and RNA pools (Fig. 17A). Phylogenetic analysis showed, that sequences related to bacteria of class *Flavobacteria* run on the above half of the gel, while *Sphingobacteria* run in the bottom-half. Bands were more numerous in the soil in comparison with the cast samples amplified after RT. Bands 1, 1/1, 2, 2/1, 3-5, 8-16 were found to be common for all investigated samples. Among them, the bands 1-5 belonged to the class *Flavobacteria*, and bands 8-18 linked to the class *Sphingobacteria*. Bands 6 and 7 appeared in the earthworm casts samples amplified after RT and presented also in all samples amplified from DNA, and related to genus *Flavobacterium* (Fig. 17B).

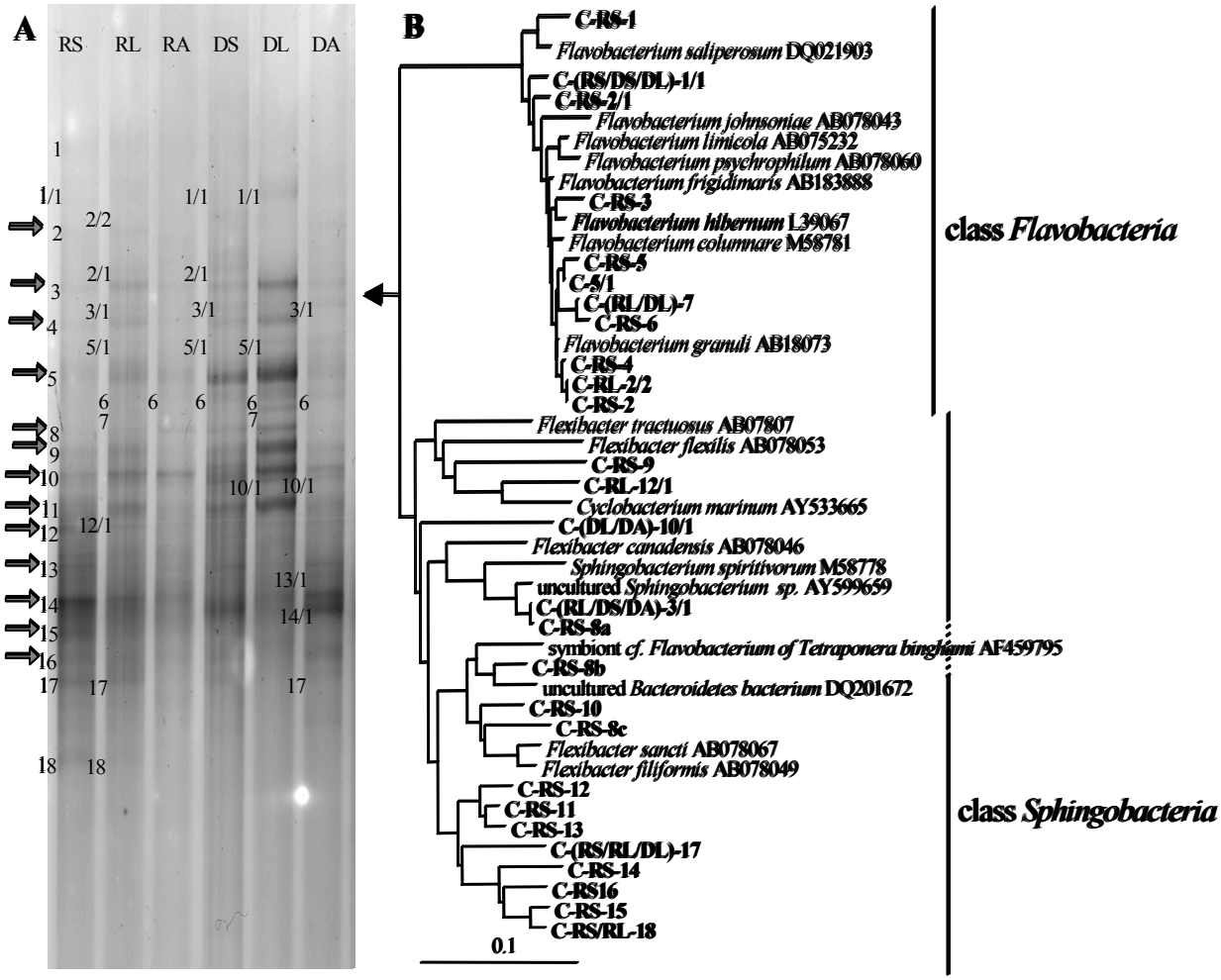


Figure 17. A: SSCP analysis of *CFB* -specific PCR products generated from total RNA (using reverse transcription) (R) and total DNA (D). Bands represented in all profiles marked with arrows. Lanes: soil (S); earthworm *L. terrestris* (L); *A. caliginosa* (A); **B**: Phylogenetic analysis of the sequences from the *CFB*-specific SSCP analysis. Several ribotypes detected in one band represented on phylogenetic tree with the number and small letters (a and b). Sequences with identity >98,5% were recognized as a single OTU and are shown as one ID.

Bacilli (Firmicutes) – specific SSCP assay

PCR product with *Bacilli*-specific primers was obtained from all RNA samples and only from one soil DNA sample. SSCP analysis showed similarity in band distribution between soil samples generated from RNA and DNA (Fig. 18A). Sequence analysis revealed non-specifically amplified *Clostridia* (*Firmicutes*) and *Myxococcales* (*Deltaproteobacteria*) (Fig. 18B). Uncultured *Firmicutes* detected with this approach strongly related to genera *Bacillus*, *Paenibacillus*, *Sporosarcina* (class *Bacilli*) and genera *Thermoactinomyces* and *Desulfosporosinus* (class *Clostridia*) (Fig. 18B).

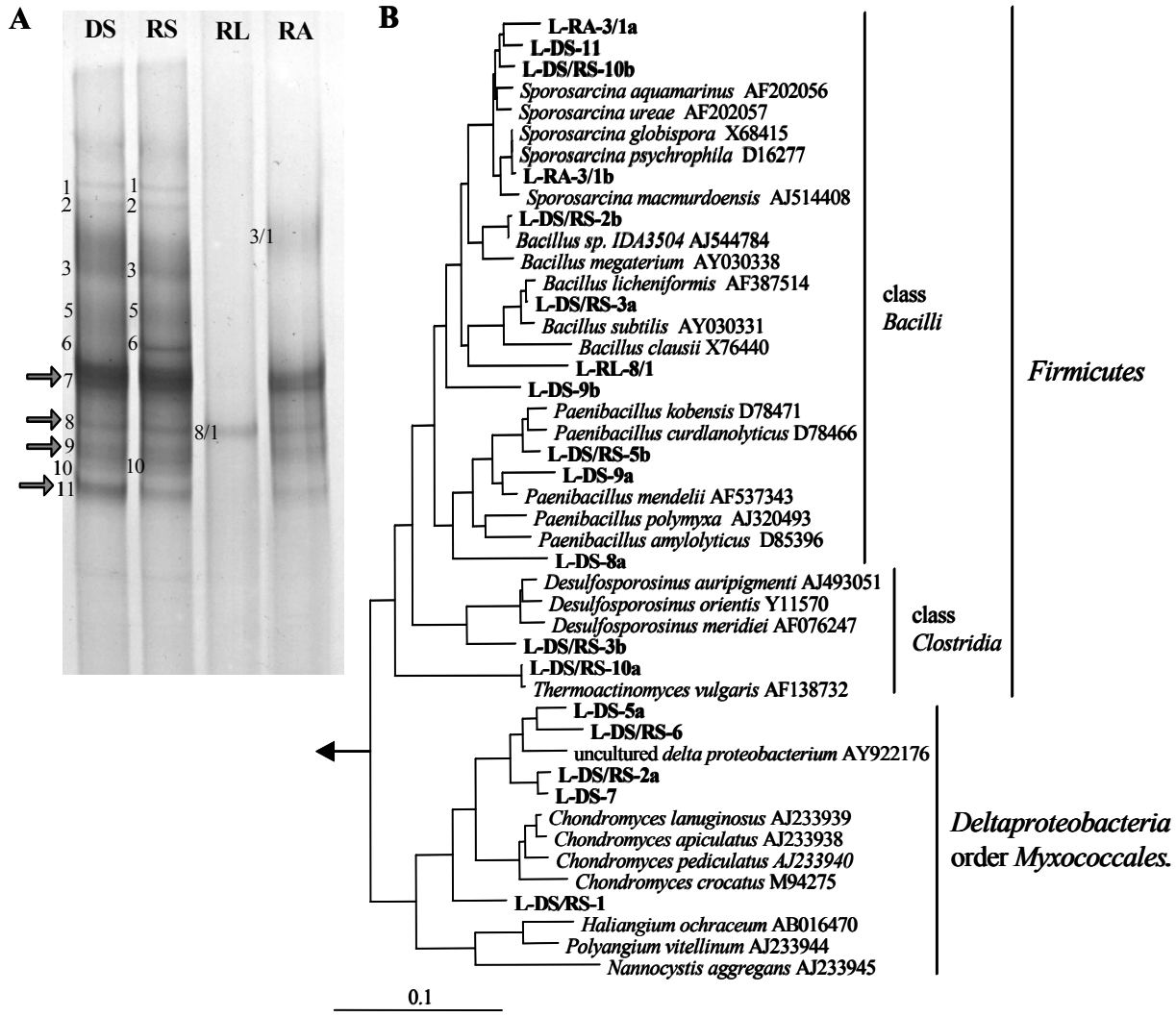


Figure 18. A: SSCP analysis of *Bacilli*-specific PCR products generated from total RNA (using reverse transcription) (R) and total DNA (D). Lanes: soil (S); earthworm *L. terrestris* (L); *A. caliginosa* (A); **B**: Phylogenetic analysis of the sequences from the *Bacilli*-specific SSCP analysis. Several ribotypes detected in one band represented on phylogenetic tree with the number and small letters (a and b). Sequences with identity >98,5% were recognized as a single OTU and are shown as one ID.

Amplification of bacteria from phyla *Verrucomicrobia*, *Planctomycetes* and class *Deltaproteobacteria* considered as minor members of bacterial communities soil and earthworm casts (according to th FISH analysis) was successfully performed only from DNA pools of corresponding samples. The diversity of those bacteria was higher in the soil, than in the cast samples from both of the earthworm species. The phylotypes had low sequence identity (<95%) to described bacterial species.

Deltaproteobacteria-specific SSCP assay

This class of *Proteobacteria* was amplified from the soil and *A. caliginosa* cast samples (Fig. 19A). Phylogenetic analysis demonstrated wide-ranging taxonomic diversity in the soil sample (the lowest sequence similarity between the ribotypes was 92%, the highest one >97%), while in the cast sample diversity was on the strain-taxonomy level (sequence identity between the ribotypes was 99%). Most of the sequences in soil and cast samples clustered together and linked to family *Polyangiaceae*. One pattern from the soil sample (D-DS-5b) was affiliate to the order *Myxococcales* with uncertain placement within this taxonomic group (Fig. 19B).

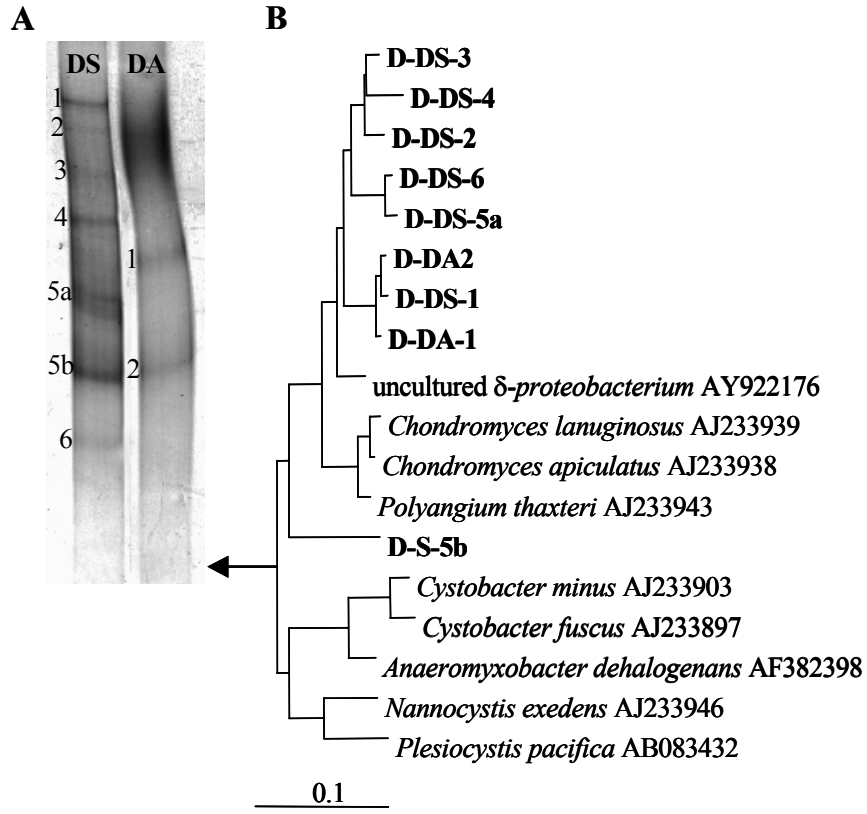


Figure 19. A: SSCP analysis of *Deltaproteobacteria*-specific PCR products generated from total RNA (using reverse transcription) (R) and total DNA (D). Lanes: soil (S); earthworm *L. terrestris* (L); *A. caliginosa* (A); **B**: Phylogenetic analysis of the sequences from the *Deltaproteobacteria*-specific SSCP analysis. Several ribotypes detected in one band represented on phylogenetic tree with the number and small letters (a and b). Sequences with identity >98,5% were recognized as a single OTU and are shown as one ID.

Verrucomicrobia-specific SSCP assay

The PCR products with *Verrucomicrobia*-specific primers were obtained from soil and earthworm cast but the size of amplified fragments was shorter (~300 bp), than expected. The patterns appeared on the gel were pretty similar each to other (Fig. 10A). Phylogenetic analysis of the sequences revealed unspecifically amplified 16S rRNA from bacteria form family *Streptomycetaceae* (*Actinobacteria*) (Fig. 20B) and wide-ranging diversity of *Verrucomicrobia* ribotypes, which were mainly linked to uncultured bacteria but some sequences clustered with *O. terrae*. The pattern V-DS-3a and V-DS-2a were similar (sequence identity 95%) to the clones detected in the *L. terrestris* libraries, and linked to *A. mucinifila*.

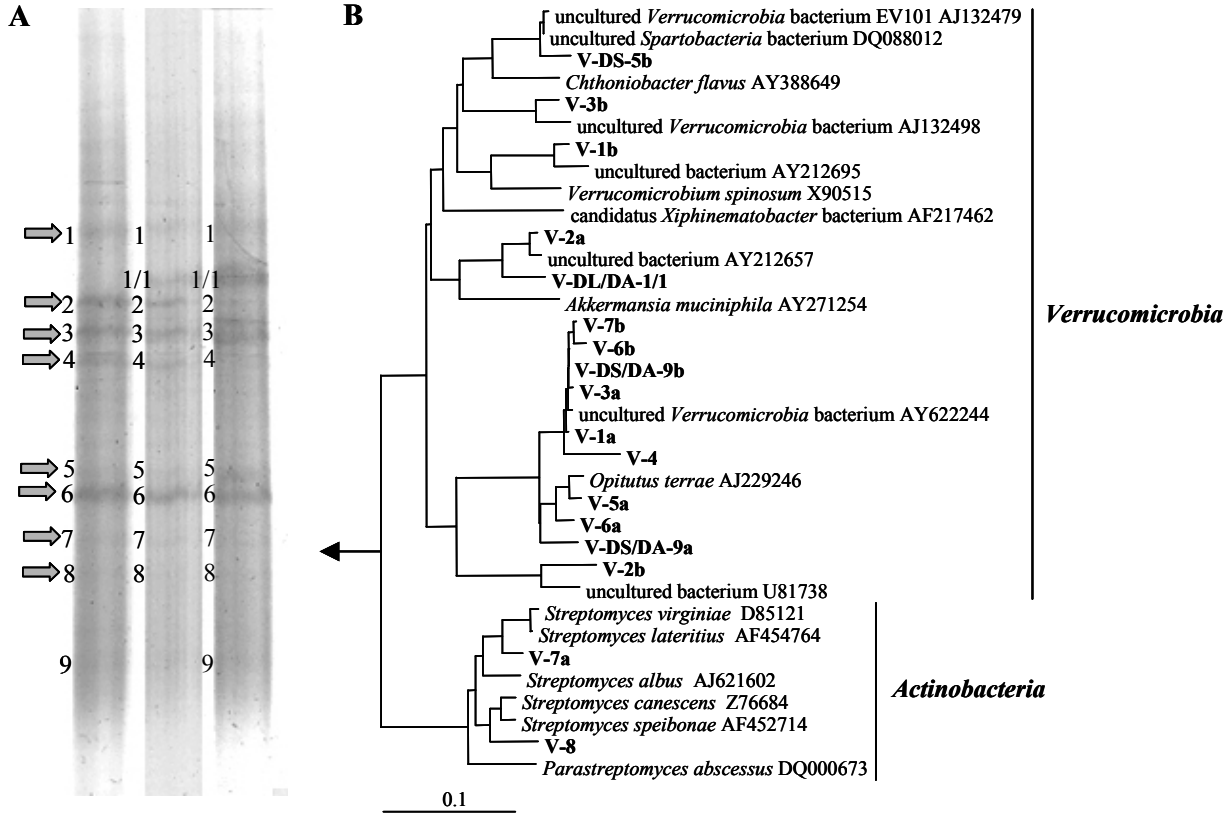


Figure 20. A: SSCP analysis of *Verrucomicrobia*-specific PCR products generated from total RNA (using reverse transcription) (R) and total DNA (D). Lanes: soil (S); earthworm *L. terrestris* (L); *A. caliginosa* (A); **B**: Phylogenetic analysis of the sequences from the *Verrucomicrobia*-specific SSCP analysis. Several ribotypes detected in one band represented on phylogenetic tree with the number and small letters (a and b). Sequences with identity >98,5% were recognized as a single OTU and are shown as one ID.

Planctomyces-specific SSCP assay

SSCP profiles of the patterns from the phylum *Planctomycetes* were comparable each to other in all estimated samples (Fig. 21A) and phylogenetic analysis confirmed specificity of the primer set and identity of the neighbor bands running in the different lines each to other. The ribotypes linked to genera *Isophaera*, *Gemmata*, *Pirellula* and *Planctomyces* without clustering (Fig. 21B).

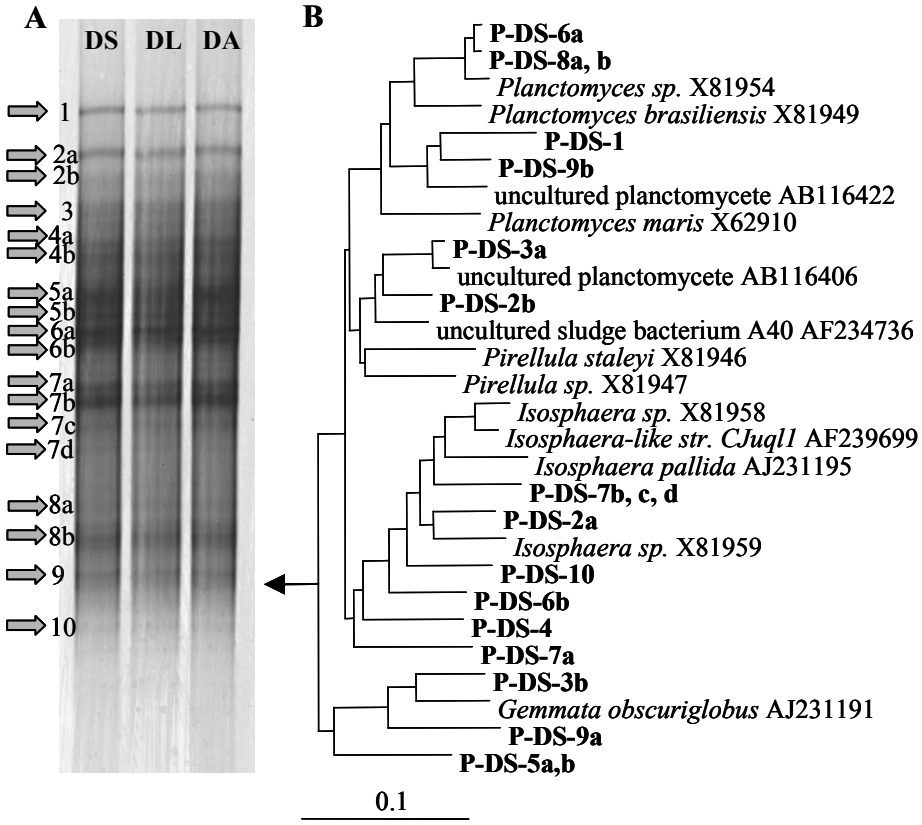


Figure 21. A: SSCP analysis of *Planctomycetes*-specific PCR products generated from total RNA (using reverse transcription) (R) and total DNA (D). Bands represented in all profiles marked with arrows. Lanes: soil (S); earthworm *L. terrestris* (L); *A. caliginosa* (A); **B**: Phylogenetic analysis of the sequences from the *Planctomycetes*-specific SSCP analysis. Several ribotypes detected in one band represented on phylogenetic tree with the number and small letters (a and b). Sequences with identity >98,5% were recognized as a single OTU and are shown as one ID.

3.2 Recombinant prion proteolysis assays

3.2.1 Proteolytic activity of pure isolates

Bacterial isolates (totally, 800) from the soil, compost and earthworm sources had generally a high sequence identity (>98%) to the taxonomically recognized organisms and only a few strains had lower sequence identity. Identification of isolates revealed a high diversity of the bacteria from clases *Gamaproteobacteria* and *Actinobacteria*, while the isolaes from *CFB* group were scarce, most likely due to the prevalently aerobic isolation approach.

Sole recPrP-digesting species were detected in the classes *Alphaproteobacteria* (*Bosea minatitlanensis* 478-1, isolated from the cast of *A. caliginosa*) and *Betaproteobacteria* (*Massilia timonae* 434-2) both of them were isolated from the cast of *A. caliginosa* (Fig. 22, 23).

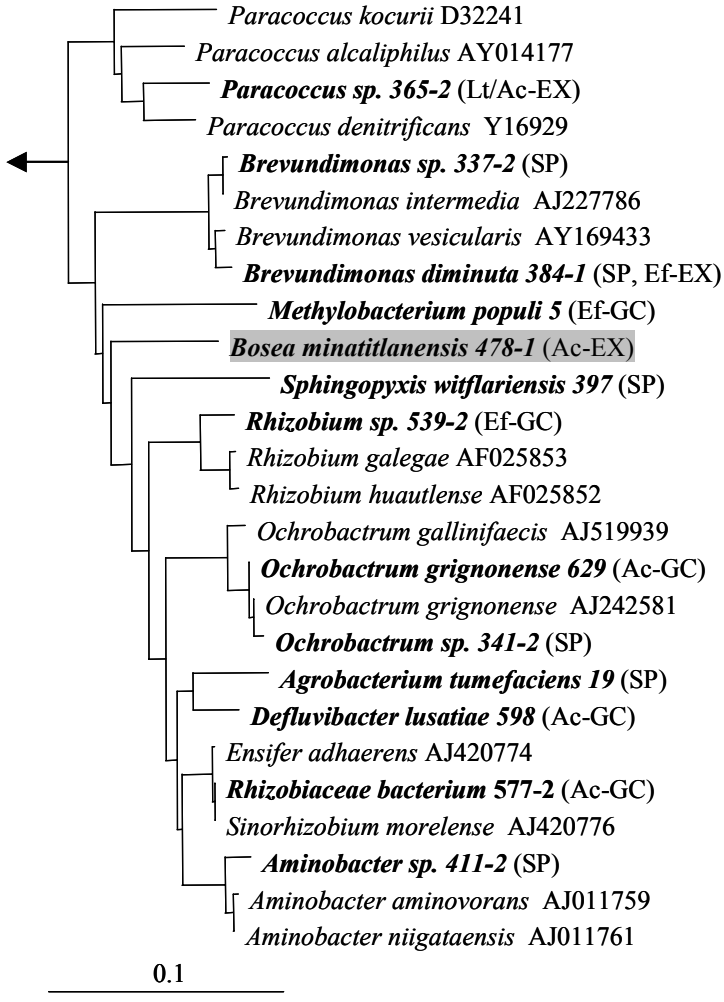


Figure 22. Bacterial isolates (marked in bolt) of the class *Alphaproteobacteria*. Bacterium in the gray box digested recPrP. The tree is constructed with sequences of ~500 bp length; the sequences with identity >98,5% to each other and to corresponded described species considered as the same OTU and presented in single example without correspondent bacteria. Sources of isolation: *L. terrestris* (Lt); *A. caliginosa* (Ac); *E. fetida* (Ef); soddy-podzolic soil (SP); gut content (GC); casts (EX).

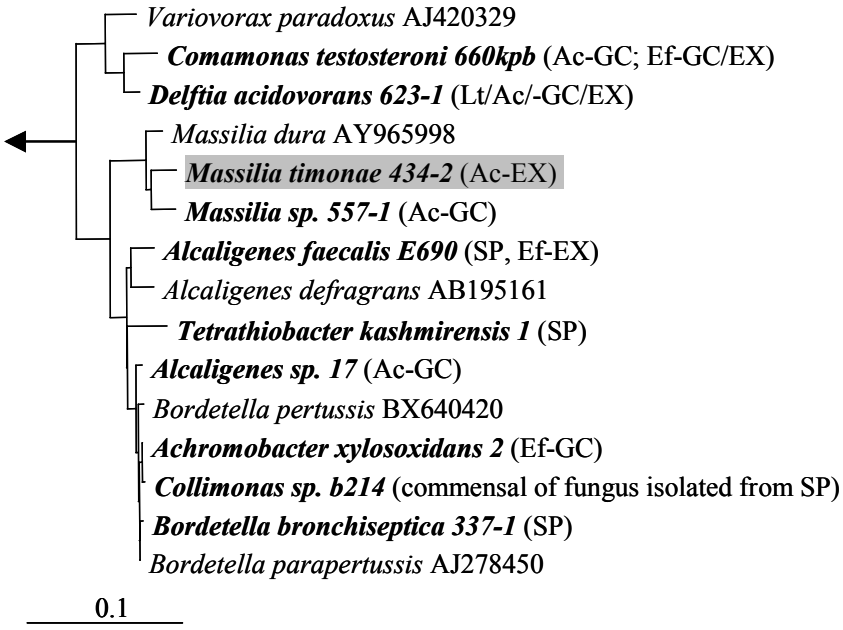


Figure 23. Bacterial isolates (marked in bolt) of the class *Betaproteobacteria*. Bacterium in the gray box digested recPrP. The tree is constructed with sequences of ~500 bp length; the sequences with identity >98,5% to each other and to corresponded described species considered as the same OTU and presented in single example without correspondent bacteria. Sources of isolation: *L. terrestris* (Lt); *A. caliginosa* (Ac); *E. fetida* (Ef); soddy-podzolic soil (SP); gut content (GC); casts (EX).

Bacterial species from the class *Gammaproteobacteria* able to digest recPrP were abundant between other *Proteobacteria*: 9 species of total 27 isolated appeared recPrP proteolytic activity. The bacteria from the genus *Pseudomonas* were the most successful (Fig. 24). The isolate *P. agglomerans* 571-1 did not digest the not-structured N-terminus of the recombinant prion (epitope of PrPc248 antibody); the isolates *A. sorbia* and *Pseudomonas sp. 9* did not degrade the interhelix loop at the C-terminus of the recPrP (epitope of VRQ14 antibody) (Fig. 24).

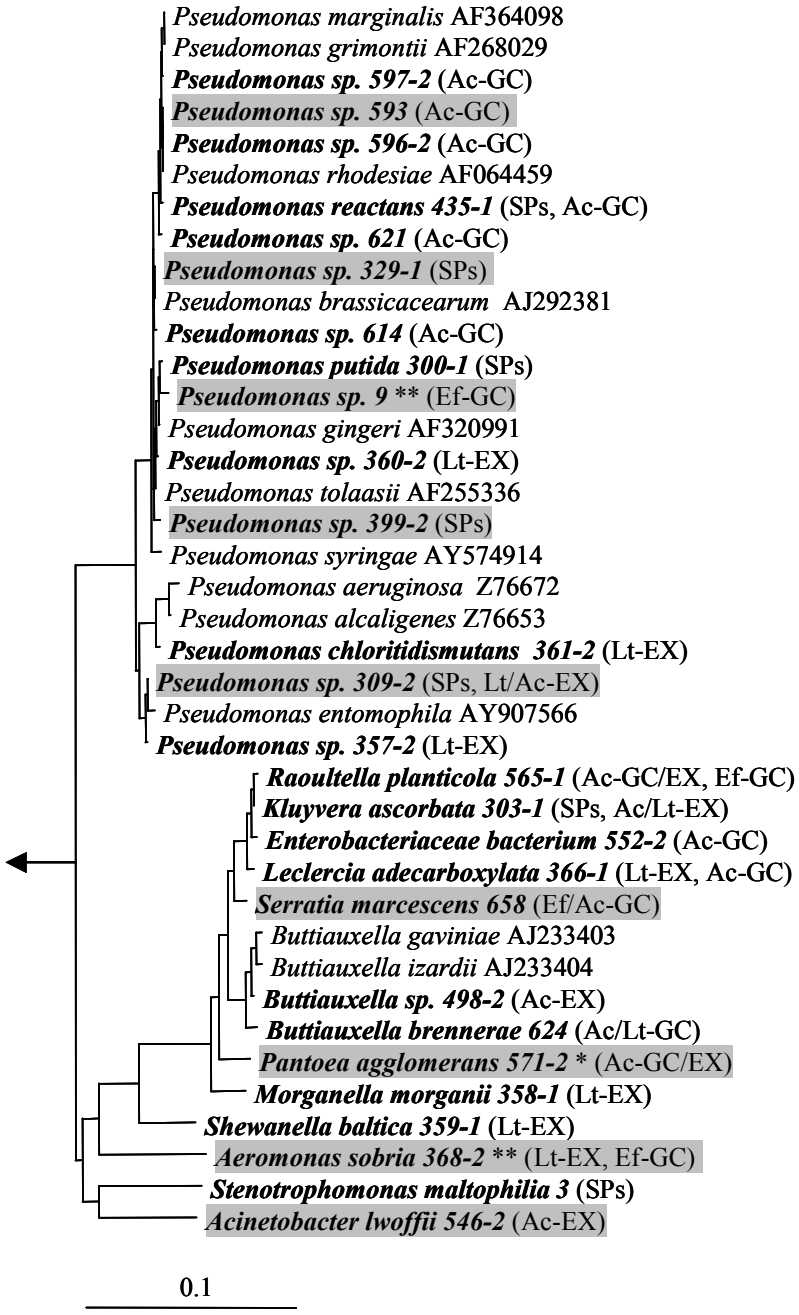


Figure 24. Bacterial isolates (marked in bolt) of the *Gammaproteobacteria* group. Bacteria in the gray box digested recPrP. * - bacterium digested only N-terminus of recPrP (epitope PrPc248); ** - bacteria digested only C-terminus of recPrP (epitope VRQ 14). The tree is constructed with sequences of ~500 bp length; the sequences with identity >98,5% to each other and to corresponded described species considered as the same OTU and presented in single example without correspondent bacteria. Sources of isolation: *L. terrestris* (Lt); *A. caliginosa* (Ac); *E. fetida* (Ef); soddy-podzolic soil (SP); gut content (GC); casts (EX).

None of estimated randomly choused isolates of the bacteria from *CFB* group did recPrP digestion on the remarkable level (Fig. 25).

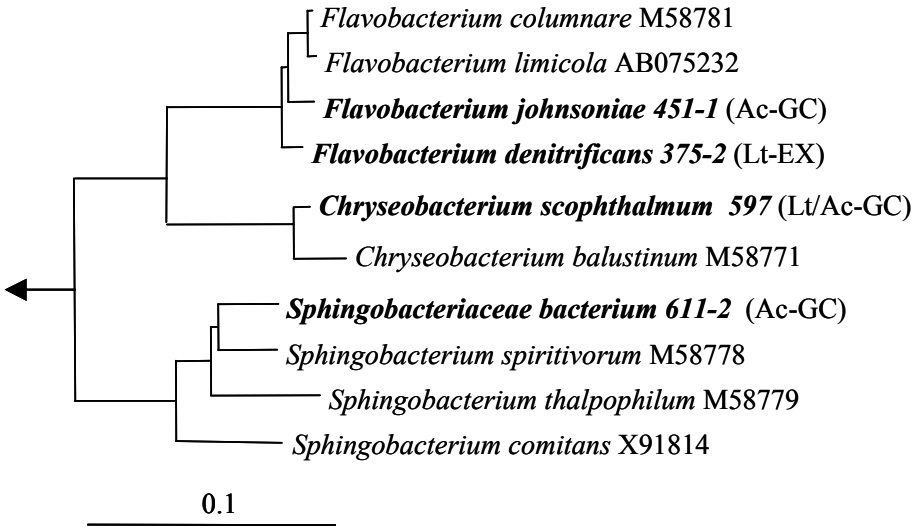


Figure 25. Bacterial isolates (marked in bolt) of the *CFB* group. Bacteria in the gray box digested recPrP. The tree is constructed with sequences of ~500 bp length; the sequences with identity >98,5% to each other and to corresponded described species considered as the same OTU and presented in single example without correspondent bacteria. Sources of isolation: *L. terrestris* (Lt); *A. caliginosa* (Ac); *E. fetida* (Ef); soddy-podzolic soil (SP); gut content (GC); casts (EX).

Isolates of the class *Actinobacteria* capable to digest recPrP were numerous as *Gammaproteobacteria*: 8 species among 30 isolated degraded recombinant prion (Fig. 26). The isolate *Nocardioides sp.* 410-1 did not digest the not-structured N-terminus of the recombinant prion (epitope of PrPc248 antibody) (Fig. 26).

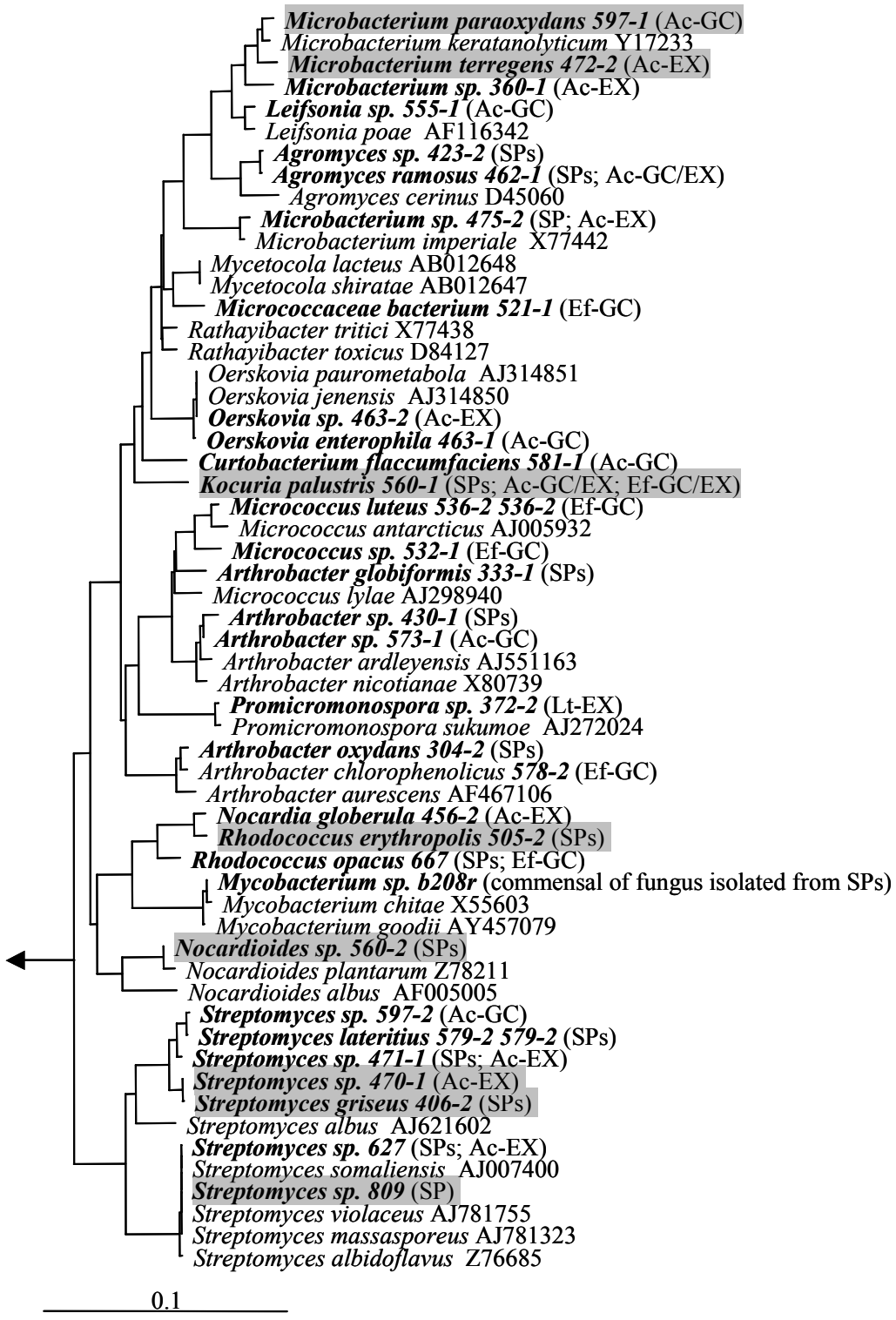


Figure 26. Bacterial isolates (marked in bolt) of the *Actinobacteria*. Bacteria in the gray box digested recPrP. The tree is constructed with sequences of ~500 bp length; the sequences with identity >98,5% to each other and to corresponded described species considered as the same OTU and presented in single example without correspondent bacteria. Sources of isolation: *L. terrestris* (Lt); *A. caliginosa* (Ac); *E. fetida* (Ef); soddy-podzolic soil (SP); gut content (GC); casts (EX).

Isolates only from the genus *Bacilli* (5 among 20 different species) appeared recPrP proteolytic capacity. The digestion by those bacteria was complete (Fig. 27).

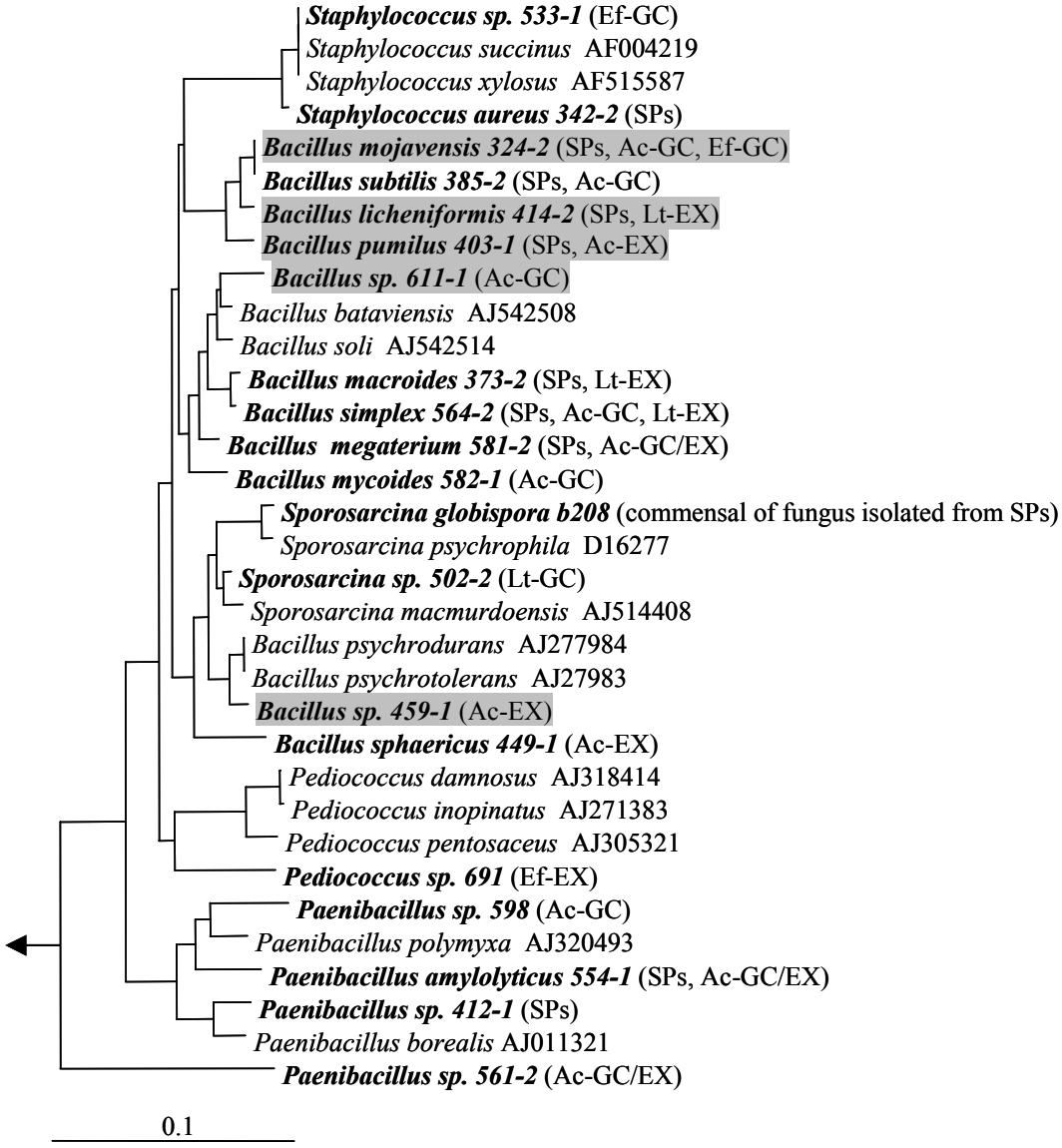


Figure 27. Bacterial isolates (marked in bolt) of the class *Bacilli*. Bacteria in the gray box digested recPrP. The tree is constructed with sequences of ~500 bp length; the sequences with identity >98,5% to each other and to corresponded described species considered as the same OTU and presented in single example without correspondent bacteria. Sources of isolation: *L. terrestris* (Lt); *A. caliginosa* (Ac); *E. fetida* (Ef); soddy-podzolic soil (SP); gut content (GC); casts (EX).

Six isolates from the most common eight fungal species were able to digest prion: *Ceriporiopsis subvermispora* (*Basidiomycota*); *Bionectria ochroleuca, Fusarium oxysporum, Tolypocladium inflatum, Gibberella sp*. (*Ascomycota*); and fungus of family *Mucoraceae* (*Zygomycota*). The residual amount of recPrP in all fungal samples was below the detection level. *Bjerkandera adusta* and *Trichosporon dulcitum* (*Basidimycota*) did not digest prion under given conditions.

3.2.2 Effect of earthworms and gut microbiota on recPrP retaining

Unspecific proteolytic activity

Recombinant PrP was not detected in the soil control samples under given extraction conditions, hence all experiments with soil and cast samples aimed at the recPrP proteolytic potential and specific enzymatic kinetics of the recPrP digestion were performed *in vitro* with aqueous extracts from soil and casts. That could be caused by the irreversible absorption of the recPrP to the soil particles (Vasina *et al.,* 2005; Johnson *et al.,* 2006).

The amount of total protein in aqueous extracts was similar each to other (2,17; 2,30; 2,23 ng/µl in the soddy-podzolic soil, *L. terrestris* and *A. caliginosa* casts respectively).

Maximal proteolytic activity was observed in all aqueous extracts without protease inhibitors (1). Inhibitors had affected the enzymatic activity of water-soluble fractions from the soil and cast samples, but proteolysis has still been performed in all variants with added inhibitors; using even two cocktail sets together (2) (Fig. 28A). Degradation of chromogenic substrate in soil extract was mostly caused by action of proteases, which were inhibited by EDTA presented in the Cocktail Set II, while the Inhibitor Set III did not affect so successfully (Fig. 28A). In contrast to the soil, proteases inhibited by aprotinine and leupeptine (presented in the Cocktail Set III) played the main proteolytic role in cast samples (Fig. 28A).

Proteinase K and trypsin did also digest commercially available chromogenic substrate differently probably because of diverse composition of substrates available for hydrolysis; PK was more efficient in proteolysis (Fig. 27B).

Non-specific proteolytic activity of water extract from soil sample, which contained 2,17 ng/µl of total soluble proteins (in 40 µl of final sample volume) without additional inhibitors, was approximately equivalent to the activity of 0,125 ng/µl proteinase K (0,15 mU). Proteolysis of chromogenic substrate in the aqueous extracts from the casts of both earthworm species, without inhibitors, was much stronger than in the soil sample and even exceeded the activity of the same amount of pure proteases: 2,30 ng/µl of total proteins in the cast sample of *L. terrestris* and 2,23 ng/µl of total proteins in the cast sample of *A. caliginosa* showed the enzymatic activity comparable to approximately 50 ng/µl (~60 mU) and 17,5 ng/µl (~20 mU) of proteinase K, respectively (Fig. 27B).

Activities of the proteases inhibited with EDTA (Inhibitor Coctail Set II) in presence of Inhibitor Set III (4) in all samples were very similar each to other and corresponded to the activity of 0,0625 ng/µl (0,075 mU) proteinase K. The activities of the proteases inhibited with aprotinin and leupeptine (Inhibitor Set III) with added Inhibitor Set II (3) were higher in the water extracts from

the casts of both earthworm species and analogous to the action of 5 ng/µl (6 mU) proteinase K in the contrast to that from the soil sample, which correlated to 0,0113 ng/µl (0,0135 mU) proteinase K (Fig. 27). According to the optical density of digested chromogenic substrate, the summarize activity of proteases in the variants with separately added Inhibitor Sets were equal to the variant with presence of the both Inhibitor Set together in the aqueous extract derived from the same sample, soil or earthworm casts (Fig. 27A).

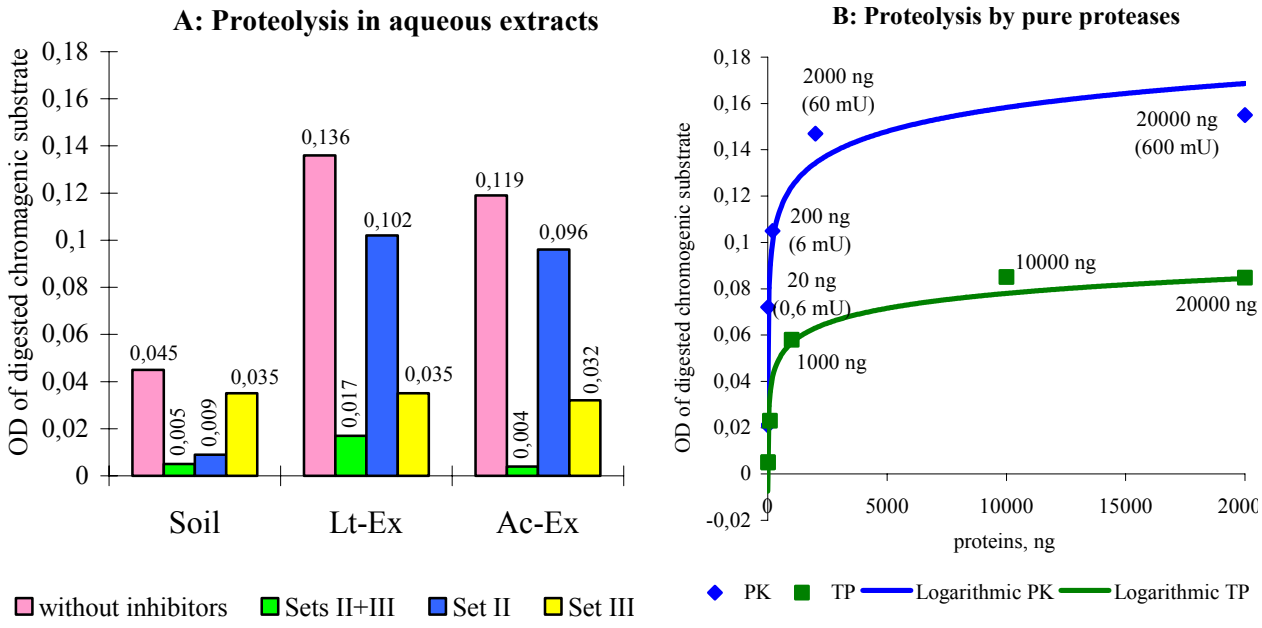


Figure 28. A: Unspecific proteolysis of chromogenic substrate (GenoTech, USA) in the aqueous extracts from soil and casts from *L. terrestris* (Lt-EX) and *A. caliginosa* (Ac-EX) with/without Protease Inhibitor Cocktail Sets; **B:** Unspecific proteolysis of chromogenic substrate (GenoTech, USA) by proteinase K (PK) and trypsin (TP).

Wide spectrum of proteases presented in the aqueous extracts and their broad specificity could caused the stronger digestion of chromogenic substrates and thus, higher optical density of reaction mixture in comparison with pure proteases. The pure proteases revealed logarithmic progression for the digestion of chromogenic substrate, while the proteases in the aqueous extracts had linear progression for the tested concentrations (data not shown).

Thus, proteases inhibited with EDTA played a major role in the non-specific proteolysis in soil aqueous extracts, and the proteases inhibited with aprotinin and leupeptine augmented upon passage through the earthworm gut caused a significantly enhanced non-specific proteolysis in water-soluble content from the earthworm casts.

Proteolysis of recPrP

Dynamics of recombinant prion degradation detected with both antibodies was similar each to other (Fig. 29, 30). Visible changes of prion amounts were already observed in the soil and cast from samples both earthworm species with bacterial inhibitor protease Cocktail Set II (3) after 12 hours (data not shown). The reduction of recPrP quantity became remarkable after 1-day incubation in the soil and cast extracts without protease inhibitors (1). Complete digestion of recPrP was observed in the samples without inhibitors (1) and with protease Inhibitor Set II contained EDTA (3) using antibody PrPc248 (Fig. 29).

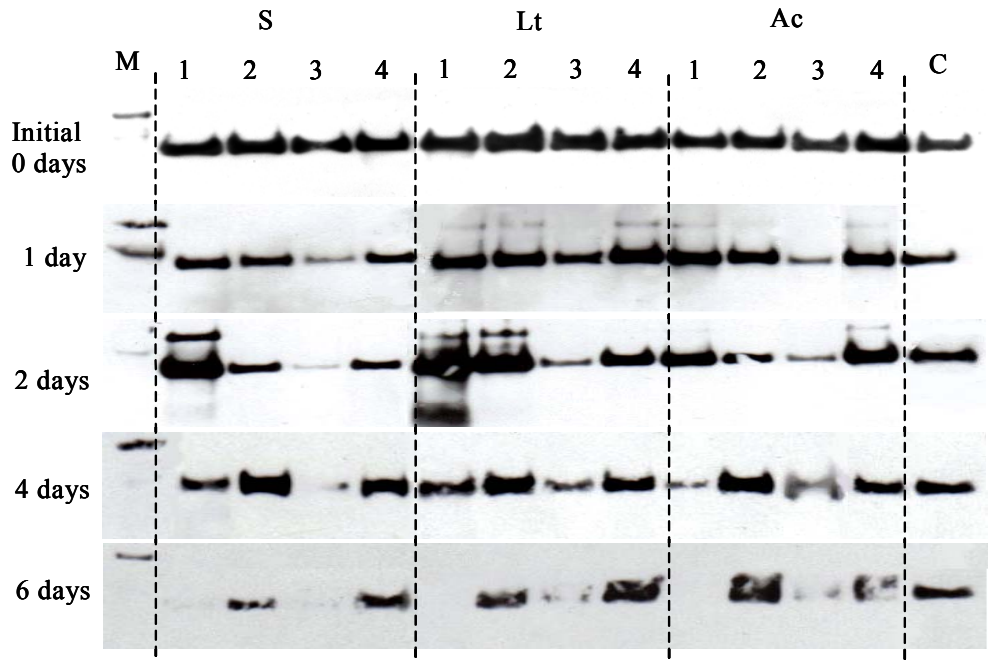


Figure 29. Immunodetection of recPrP with the antibody PrPc248 after incubation of the recPrP for 0, 1, 2, 3, and 6 days at 15° C with the aqueous extracts from soddy-podzolic soil (S), cast of *L. terrestris* (Lt) and *A. caliginosa* (Ac) detected Lanes: MultiMark Multi Colored Standard (M); control, 40 ng (C); without inhibitors (1); with protease Inhibitor Cocktail Sets (II+III) (2); with protease Inhibitor Cocktail Set II (3); with protease Inhibitor Cocktail Set III (4).

Degradation of recombinant prion detected with antibody VRQ 14 was also more rapid in presence of Inhibitor Set II (3) (Fig. 30).

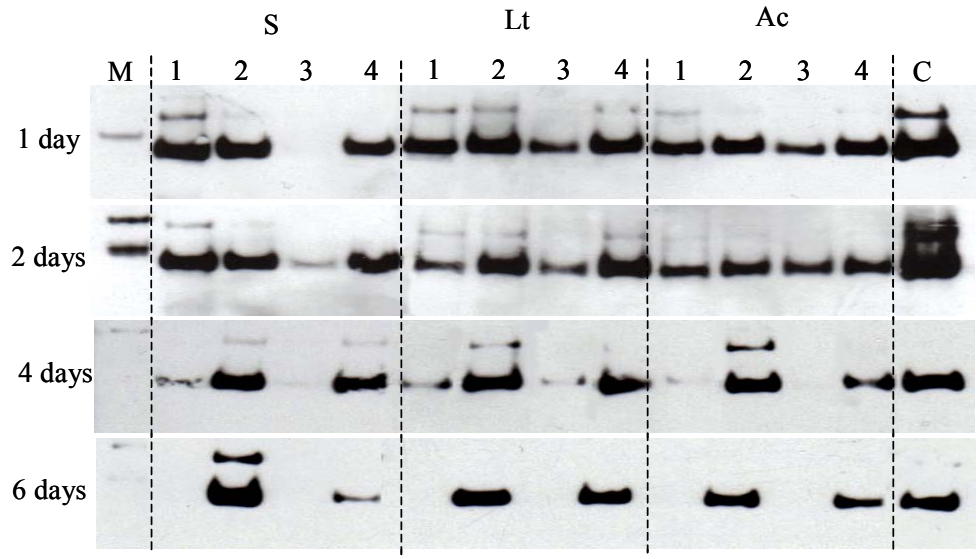


Figure 30. Immunodetection of recPrP with the antibody VRQ14 after incubation of the recPrP for 0, 1, 2, 3, and 6 days at 15° C with the aqueous extracts from soddy-podzolic soil (S), cast of *L. terrestris* (Lt) and *A. caliginosa* (Ac) detected Lanes: MultiMark Multi Colored Standard (M); control, 40 ng (C); without inhibitors (1); with protease Inhibitor Cocktail Sets (II+III) (2); with protease Inhibitor Cocktail Set II (3); with protease Inhibitor Cocktail Set III (4).

Notable prion digestion was observed neither in presence of both Inhibitor Cocktail Sets together (2), nor with Inhibitor Cocktail Sets III (4) during the whole term of incubation with both used antibodies (Fig 29, 30).

In general, the proteolytic property in aqueous extracts from soil and cast samples did not show remarkable differences between for prion hydrolysis in contrast to unspecific proteolytic activity.

Pure trypsin (0,8 ng/µl) did not digest ovine recPrP during 6-days incubation time (Fig. 31), while α-chymotrypsin and proteinase K lead to a rapid (within 15 min) hydrolysis of the full-length recombinant prion (Rezaei *et al.,* 2000).

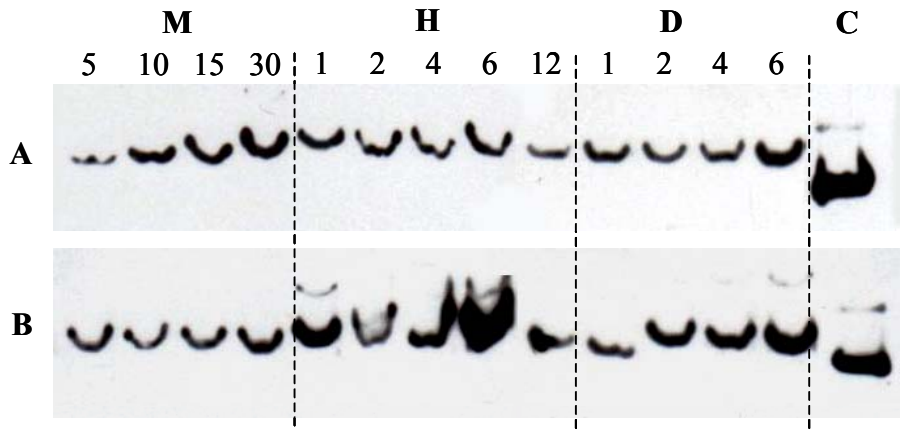


Figure 31. Immunobloting of recPrP treated with pure trypsin detected by PrPc248 (A) and VRQ 14 (B) antibodies after incubation for: 5, 10, 15, 30 minutes (M); 1, 2, 4, 6, 12 hours (H); and 1, 2, 4, 6 days at 15° C in comparison with control (C) recPrP (40 ng).

4. Discussion

4.1 Effect of the earthworm gut environment on the microbial community

The changes of bacterial community of soil or compost upon passage through the earthworm GI tract were studied using earthworm species populating soddy-podzolic soil (*L. terrestris* and *A. caliginosa*) and horse manure compost (*E. fetida*). The experimental setup exploited: (i) different earthworm species (*L. terrestris* and *A. caliginosa*) thriving in the same substratum (soddy-podzolic soil) and the same species of the earthworm from the same soil type but with another bacterial community and (ii) the earthworms (*E. fetida*) populating quite different substratum (horse manure compost).

On the basis of the FISH analysis we consider that bacterial communities of studied substrata (soil and compost) were significantly modified passing through the gut of earthworms. The ratio EUB338/DAPI (illustrated the percentage of physiologically active cells) for the soddy-podzolic soil collected at different years was comparable each to other (0,18-0,20), while the index was higher in the cast samples of earthworms (*L. terrestris* (0,27-0,29) and *A. caliginosa* (0,23-0,30)) . At the same time the index EUB338/DAPI was lower in the cast of earthworm *E. fetida* in comparison with the horse manure compost. This proportion of the cells hybridized with FISH probe to those stained with DAPI had the lowest number in the guts of estimated earthworms (excluding *A. caliginosa* collected at the spring 2003). In general, the ratio EUB338/DAPI was pretty similar to those described early by Schönholzer and colleagues (2002) for soil and earthworm *L. terrestris*. Augmentation with bacteria during transit was also detected elsewhere with plating method (Daniel and Anderson, 1992) and epifluorescence microscopy (Kristufek *et al.,* 1992).

Besides, almost all taxonomic groups were undergoing significant changes. The alterations in microbial composition exhibited some commonalities in the earthworms of different species. As such one should notice: (i) augmentation of *Cytophaga-Flavobacteria* group of *Bacteroidetes* upon passage in all our independent experiments throughout all three years of the present study; (ii) numbers of *Firmicutes* and *Gammaproteobacteria* were in most cases lower in gut content in contrast to substratum; (iii) microbial composition alterations of *Actinobacteria*, *Alpha*- and *Betaproteobacteria* varied and could increase or decrease upon passage; (iv) bacteria of phyla *Planctomycetes*, *Verrucomicrobia* and *Deltaproteobacteria* had very low numbers (< 1%) of physiologically active cells in population being minor members of community, furthermore the passage through the earthworm gut did not notably effect them.

Another issue we tried to address was whether a common regularity of changes of microbial community upon its passage through the GI tract of earthworms does exist and whether there is the host or substratum dependence influencing these changes. Same major patterns in microbial community composition changes were observed for two different earthworm species (*L. terrestris* and *A. caliginosa*) populating the same soil with the same microbial inocula and containing in contrast to those of a same species kept in the soils with different initial microbial compositions.

All these facts indicate that microbial composition of inoculum (microbial biodiversity in the soil/compost) was the major factor affecting the further population changes during the passage through the earthworm gut; species-specific features of the earthworm gut environment affecting the bacterial population changes upon passage were not observed.

Among the physical-chemical factors in the earthworm gut the following items are known: (i) anoxic conditions (Karsten, Drake, 1995; Horn *et. al.* 2003); (ii) higher (in comparison with soil) content of water, organic and amino acids, sugars, nitrite, ammonium and hydrogen (Karsten, Drake, 1995; Horn *et. al*. 2003); (iii) mechanical (Schönholzer *et al*., 2002) and biochemical disintegration of microorganisms in the digestive tract of earthworms (Edwards and Fletcher, 1988), killing activity of gut exudates was noticed for soil diplopods and millipedes (Byzov *et al*., 1996, 1998); (iv) interaction of microorganisms between each other (including the whole spectrum of possibilities, from antagonism to mutualism) (Schönholzer *et al*., 2002).

Thus, earthworm gut environment has a strong selective pressure influences certain bacterial taxonomy groups being a “bottleneck” with unfavorable conditions decreasing total number of certain physiologically active microbial groups hybridized with FISH probes. At the same time, the very conditions, inhibiting or even deadly to some members from bacterial population, stimulate other bacteria flourishing in the gut or in the cast. Similar qualitative variation patterns of the same taxonomic bacterial groups upon passage through the guts of *A. caliginosa*, *L. terrestris* and E. fetida inhabiting various substrata with different bacterial communities were observed and were possibly caused by similar selective pressure factors of gut environments of earthworms.

One of the most challenging questions in the earthworm microbiology is what kind of organisms we can consider as intestine symbiotic or gut-associated. There are two possible definitions: (i) obligate gut-associated microorganisms cannot survive out of earthworm gut or their numbers are getting extremely low and they become in physiologically inactive; (ii) facultative gut-associated microorganisms which can survive outside the gut where they can physiologically be active, but their numbers are lower in comparison with gut lumen, and during the passage through the gut a certain enrichment of these microbes occurs. According to the FISH analysis, the bacteria of *CFB*

satisfactory fit to the definition and could be considered as facultative gut-associated microoragnisms.

Simultaneously, taxonomic groups reducing their numbers in the gut could contain certain bacterial species believed to be the gut-associated bacteria but undistinguishable in the total pool of microbes, i.e. for whom the FISH approach is too insensitive. To qualitatively evaluate bacterial communities' compositions and to determine gut-associated bacteria we have applied PCR-based methods.

Phylogenetic analysis of the clone libraries showed a high diversity of microorganisms (especially in compost), but the number of sequenced clones was relatively low and allowed just to roughly estimate bacteria populating the substrates and earthworm sources. Application of the cloning technique allows detection of even a single copy (although the probability of that for randomly chosen colonies is very low). This could be an explanation for finding in the libraries from earthworm sources (gut content and cast but not in the substrata) clones belonging to the minor part of population.

SSCP coupled with the consequent phylogenetic analysis of separate DNA bands delivers even though superficial, but important and visible information about bacterial community composition. Disadvantage of this method is based on the staining the DNA bands, which, to become visible, should harbour quite a number of DNA copies per band, and which could then be detected and analysed by re-amplification and sequencing.

In general, taxon-specific SSCP approach allows detection of bacteria on the levels of the genus, species and strain, bringing up a higher diversity of bacteria in comparison with universal SSCP primers designed by Schweiger and Tebbe (1998). Primer sets designed for detection of bacteria from classes *Alphaproteobacteria*, *Bacilli* (*Firmicutes*), and *Verrucomicrobia* appeared to possess some minor unspecific affinity with outgrouped bacteria; *Gammaproteobacteria*-specific primers annealed with unclear taxonomy-placed bacteria equally affiliated to γ- or β-proteobacteria. That could be caused by humid acid co-extracted with nucleic acid and decreased the primer specificity (Stach *et al.,* 2001).

Despite of mentioned above shortcomings, the taxon-specific SSCP worked out significantly more information than conventional SSCP. Firstly, the number of distinguishable bands obtained with universal SSCP primers (e.g. 13 bands in the sample generated from soil RNA) was notably lower than sum of the bands detected by using taxon-specific SSCP primers (90 distinguishable bands in all taxon-specific bands generated from soil RNA). Gram-negative microorganisms delivered the most of the bands on the SSCP profile. Among them, bands of the bacteria from the phyla *CFB* and

Proteobacteria were the most abundant. Despite of this, diversity of such bacteria estimated with taxon-specific primers was higher. The phyla *Firmicutes, Planctomycetes* and *Verrucomocrobia* were detected using the universal primer set neither from DNA, nor from RNA pools, while their diversity was accessed using only taxon-specific SSCP primers. Simultaneously, the bacteria from the phyla *Acidobacteria* and *Gemmatinonadetes* were detected only with universal SSCP primers for 16S rDNA gene. Thus, one may consider Gram-negative (easy-lysed) bacteria with several ribosomal operons in genome to have more chances to be detected using PCR. Perhaps this is the reason of the presence of a relatively high number of bands from scanty *Gammaproteobacteria* on the SSCP profiles generated with universal primers, which outcompete the Gram-positive bacteria for oligonucleotides and other PCR reaction ingredients which, in turn, lead to the absence of the bands corresponding to the latter organisms on the SSCP profiles generated with universal primers. Another point to mention is a moderate, comparable number of the bands on the profiles generated with universal primers (up to 15 bands per sample) and with taxon-specific primers (up to 20 – *Gammaproteobacteria* generated from total soil RNA) diverged within the similar level of magnitudes.

As far as number of physiologically inactive bacteria presented in the community was 5 times higher than active cells (i.e. the cells hybridized with unspecific FISH probe), one could expect higher number of bands on SSCP profiles generated from DNA. Inactive bacteria present in the community and "waiting" for favorable conditions to initiate their growth can be accumulated in the soil or upon the income from other sources (for instance from leaf litter) or through seasonal changes when suddenly an enormous number of species become inactive (Torsvik *et al.* 1990; Trüper, 1992). Truly, composition of bacterial community estimated with taxon-specific primers was significantly depended on the type of template (DNA or RNA). Minor members of bacterial community (*Planctomycetes*, *Verrucomicrobia* and class *Myxococcales* of *Deltaproteobacteria*) were amplified only from total DNA but not from RNA preps. Probably, number of the target 16S rRNA in the total RNA pool was below the sensitivity of the method, or one of the primers was exhausted by a non-specific binding to ribosomal RNAs from outgrouping microorganisms during reverse transcription. The single bands linked to *A. faecalis* were detected in the SSCP profiles generated from RNA pool by using *Betaproteobacteria*-specific primers along with many bands in the DNA-generated profiles. But the profiles amplified from DNA pool were not always rich with bands in comparison with those done from RNA. *Gammaproteobacteria* and *Bacilli* demonstrated an opposite picture: PCR products were obtained from the RNA, but not from DNA (casts of *L. terrestris* and *A. caliginosa*), or profile was more complex (*Gammaproteobacteria*, soil sample). High amount of total DNA could block the amplification because of excesss of the target 16S rDNA

template in case of using total DNA as template; dilution of the template could remove or reduce diversity of the target gene in the pool, caused low diversity of amplicons. The use of RT-PCR with taxon-specific primers enhanced in some cases the sensitivity of the method whereas only rRNA was amplified and thus the competition by competing molecules was omitted.

Identity of SSCP profiles in the soil and cast samples generated from DNA could be explained with the presence of the same bacteria in both samples that remain intact upon passage, which affected only the physiologically active part of population. This was consistent with the early results of Egert and colleagues (2004), which could not find dominant indigenous microbes in the gut of *L. terrestris* using T-RFLP analysis using DNA as template.

According to the published data, the 16S RNA is moderately stable in the dead cells (in comparison with even more stable DNA and sensitive mRNA) and could be detected with both FISH and RT-PCR techniques even some time after the cells die (Sheridan *et al.,* 1998; McKillip *et al.,* 1998; Lleó *et al.,* 2000; Keer and Birch, 2003). Nevertheless, the application of the 16S rRNA-based methods for studying microbial communities of substrates and earthworm casts make sense and differences between biodiversity of the samples generated from DNA and RNA were in fact very clear. Thus, comparison the data obtained with PCR using DNA as a template with those based on rRNA-based techniques (FISH) is believed to be pretty sophisticated.

Another task for the study microbial communities passing through the earthworm gut were the discovering bacterial genera and species permanently or transiently associated with this environment. Novel bands appeared on the SSCP profiles of cast samples generated from the RNA could be considered as belonged to earthworm gut-associated bacteria. These new bands were detected on the *CFB*, *Alpha-*, *Gammaproteobacteria*, and *Bacilli* taxon-specific SSCP profiles. However, do these bands really belong to bacteria coupled with earthworm intestine or occur through the possible PCR amplification errors?

The novel bands from *CFB* bacteria derived from the same species, *F. granuli,* occurring as neighbor bands, exhibited high homology degree (sequence identity 99%) each to other and formed a monophyletic cluster. Simultaneously, bacteria of the *CFB* group can thus be considered as the main candidates to form a stable association with the gut environment. The assumption that the novel band provided the growing number of *CFB* bacteria in the cast is supposed to be unrealistic. Most probably, the members of the *CFB* monophyletic cluster all together became more abundant upon the passage.

The novel bands appeared on the *Gammaproteobacteria*- and *Bacilli*-specific SSCP profiles from cast samples belonged to bacteria being a novel taxon clustering next to genera *Legionella* and *Stenotrophomonas* (*Gammaproteobacteria*) and to *Bacillus* and *Sporosarcina* (*Bacilli*).
In the contrary to other bacterial groups, almost all community of *Alphaproteobacteria* (detected on taxon-specific SSCP profile) was composed of unique bands linked to family unclassified *Sphingomonadaceae* in the soil and cast samples, although this part of total bacterial population as well as *Gammaproteobacteria* and *Firmicutes* had a tendency to reduce their number upon the passage.
On the basis of FISH, clone libraries and SSCP methods, one could distinguish three bacterial groups and approximately predict the effect of earthworm gut environment upon the microbial community of substrate (i) bacteria always increasing their numbers in the gut: classes *Flavobacteria* and *Sphingobacteria* (*Cytophaga-Flavobacteria* group of *Bacteroidetes*) was the sole bacterial group, which always benefited from the gut environment, being facultative earthworm gut-associated; (ii) bacteria always decreasing their numbers in the gut, bacteria from taxonomy groups *Gammaproteobacteria* and *Firmicutes*; (iii) bacteria without clear changes in the numbers, normally; minor members of community (*Planctomycetes*, *Verrucomicrobia* and *Deltaproteobacteria*), which have number of active cell less than 1% of the total population and were abundant neither in the soil, nor in the earthworm gut environments. The rest of bacterial taxonomy groups (*Alpha*-, *Betaproteobacteria*, and *Actinobacteria*) had varying response upon the transit depending on many factors including earthworm-bacteria and bacteria-bacteria interaction.

Particularly describing the changes of microbial community estimated with FISH and SSCP approaches one could distinguish the bacteria resistant to earthworm gut environment among the gut-sensitive taxonomy groups. Active parts of *Alphaproteobacteria* and *Betaproteobacteria* in the community detected with the SSCP were not very diverse. Although many unique bands were present on the *Alphaproteobacteria*-specific SSCP profile, they belonged only to family unclassified *Sphingomonadaceae,* while other families from the taxa (*Sphingomonadaceae Caulobacteraceae*, and *Methylobacteraceae*) were detected on the profiles generated from the DNA. The bacteria from the family of unclassified *Spingomonas* (*Alphaproteobacteria*), *A. faecalis* (*Betaproteobacteria*) and the bacteria linked to *P. enthomophila* and *P. putida* (*Gammaproteobacteria*) could be considered as earthworm gut-resistant bacteria. This statement is well supported with data from FISH analysis: *A. faecalis* seeming to be predominant bacterium was not influenced by the selective pressure of the gut environment and was stable or just slightly fluctuated upon the passage which is reflected by stable numbers of *Betaproteobacteria* in the

community. The decreasing number of *Gammaproteobacteria* was caused by strong diminishing or elimination of genera *Cellvibrio*, *Chromatiaceae,* family *Legionellaceae* from active part of community, but whether the novel bands appeared on the *Gammaproteobacteria*-specific profile truly belonged to original gut microflora is uncertain. Unclassified *Spingomonadaceae* reduced their amount being nevertheless resistant to the earthworm gut environment among the other families of the class *Alphaproteobacteria*. *Firmicutes* had a similar behavior as *Gammaproteobacteria*.

Non-diverse active part of *Proteobacteria* and other taxonomy groups together with high diversity and number of hypothetical gut-associated bacteria from *CFB* group in the soil community could be caused by a long-term-keeping (>3 months) the earthworm on the same soil with following fractional substitution of bacterial population under the earthworm gut selective pressure.

The favorable growth of *Cytophaga-Flavobacteria* cluster in the community passing the earthworm gut was previously reported for *L. terrestris* (Schönholzer *et al*., 2002) and termite gut (Schmitt-Wagner *et al.,* 2003). The bacteria from our sources were closely related to those being intracellular symbionts of the ants *Tetraponera binghami* (van Borm *et al.,* 2002b). The bacteria from *CFB* group were also detected in the gut of *Acromyrmex* leafcutter ants (van Borm *et al.,* 2002a), and other insects (Jucci, 1952). The *CFB* division is also suggested to be predominantly associated with mammals' intestine, distinct subgroup of the division being ancient and coevolved with their hosts (Bäckhed *et al.,* 2005). Reducing numbers of *Proteobacteria* and *Firmicutes* in our study well correlated with previously described data of plating/culturing methods and FISH analysis for the earthworms *Lumbricus spp.* (Pedersen and Hendriksen, 1993; Kristufek *et al.,* 1993; Schönholzer *et al*., 2002). At the same time *Actinobacteria* were not detected by FISH analysis in the gut content and excrements in that study although they were observed both in the soil and earthworm sources during our studies.

In contrast to the previously published data, scanty bacterial clones (two OTUs) from the phylum *Acidobacteria* (*Fibrobacteres/Acidobacteria* group) found in the cast library of autumn-collected *A. caliginosa* during our investigation were reported as dominant bacterial group of *L. rubellus* intestine (Singleton *et al*., 2003).

Bacteria from *Verrucomicrobia* detected on the SSCP profile from cast samples of *L. terrestris* and in the libraries from gut content and cast the same earthworm species were similar each to other (sequence identity 95%) and related to *Akkermansia muciniphila* mucosa-populating bacteria isolated from the human gut (Derrien *et al.,* 2004), hence they could be an ecological and physiological analog of this microorganism.

The increasing number of bacteria upon passage with reduction of the population of some *Gammaproteobacteria* from family *Enterobacteriaceae* well correlated with many previous studies done with the plating methods.

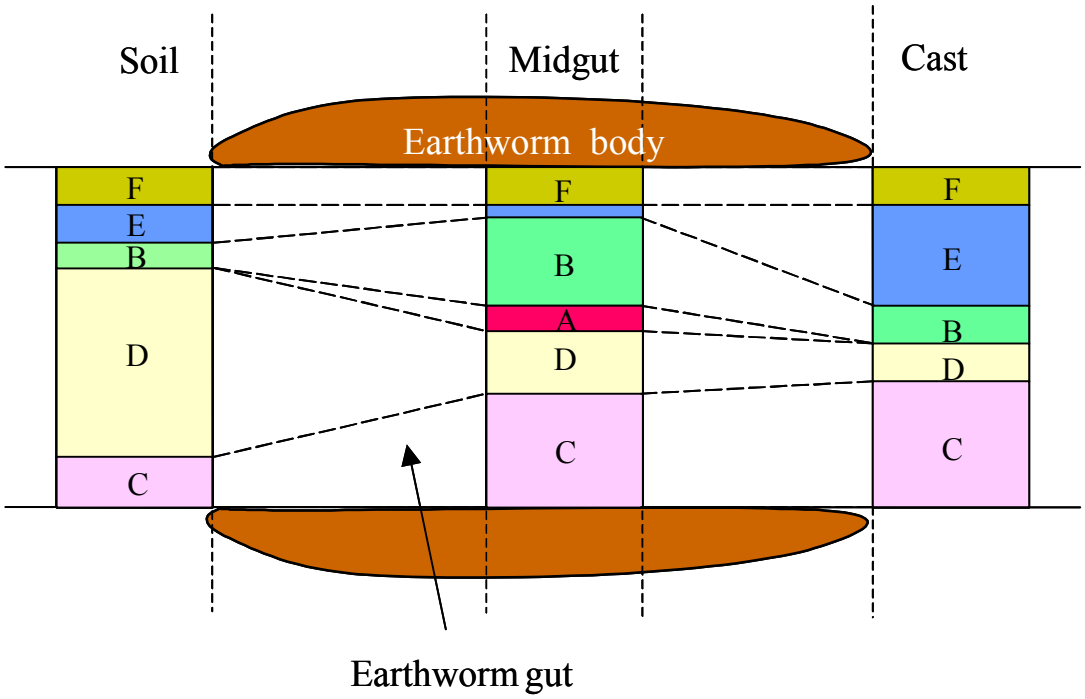


Figure 31. The scheme of changes of populations different microbial group in substratum upon passage through the earthworm gut: (**A**) obligate host-associated microorganisms ("*Lumbricoplasma*"); (**B, C**) facultative gut-associated microorganisms (*CFB* group); (**D, E**) gut-sensitive microorganisms (particularly *Gammaproteobacteria*, *Bacilli*, and *Eukarya*); (**F**) gut-resistant microorganisms.

Apart from the free-living microorganisms populating the substrate we detected certain bacteria, which could be intra- or extracellular symbionts of the earthworm. As obligate earthworm-associated bacteria one can consider bacteria from a cluster from the class *Mollicutes* (*Firmicutes*) detected in the earthworms of family *Lumbricidae* (*L. terrestris*, *A. caliginosa*, and *E. fetida*). On the basis of sequence analysis, source of isolation, and host specificity, the bacteria from newly discovered taxonomic monophyletic cluster were named '*Lumbricoplasma'* candidate division and are thought to compise a novel family, '*Lumbricoplasmataceae'*. The '*Lumbricoplasma' spp*. were detected in the gut tissue, coelom intestine, muscles, and outer epidermis being located in these tissues as small clusters. This is the first finding of this group of *Mollicutes* in the earthworm. Although the *Mycoplasma*-like bacteria were discovered by single clone in the intestine library of *L. rubellus* (Singleton *et al.,* 2003), the sequence identity with both described *Mollicutes* and '*Lumbricoplasma'* candidates was low.

Free-living amoebae harbored bacterial symbionts, which include α–, β-, γ-proteobacteria, *Flavobacteria* and *Actinobacteria*, as postulated before (Kwaik *et al.,* 1998; Greub and Raoult,

2004; Horn and Wagner, 2004; Molmeret *et al.,* 2005). Even though we detected no amoebae in our sources even accidentally, as other eukarya, many bacteria detected in our sources were related to microbes from this group. Bacteria of the genus *Legionella* populate aquatic environments but two matching clones in the *L. terrestris* sources (gut content and cast libraries) and several bands from *Gammaproteobacteria*-specific SSCP profile of soil sample related to that genus (sequence identity 92-96%); single OTU (band G-RA-33b) from the *A. caliginosa* cast sample linked to the genus *Legionella* not closely (sequence identity 87%). Besides, certain clones from the compost and earthworm *A. caliginosa* cast libraries linked to family *Anaplasmataceae*, which also included symbionts of eukarya.

Free-living amoebae could serve as vehicles for obligate intracellular bacteria and as "Troyan horse" in pathogenesis of animals (Barker and Brown, 1994). Amoebae and fungi were considered as the main nutrient sources for earthworms (Edwards and Fletcher, 1988; Brown, 1995). Hence, symbionts of amoebae are hypothetically able to change the host passing through the gut of earthworms: symbiotic bacteria released in the gut from digested protozoa could infect intestinal gut eucarya (for example roundworms detected in the gut of the earthworms) or earthworm itself and grow massively inside of new host. It was reported that earthworms might become a vector for mycobacteria (Fisher *et al.,* 2002). Consequently, the symbiotic organisms of amoebae and other protozoa could be judged as additional subsystem of microbial community of substrate incoming into the earthworm gut.

Throughout our study we have detected some differences in microbial composition of gut wall and gut content. Spatial dissimilarities in the gut microenvironments could be a possible explanation of certain augmentation with particular gut-associated microorganisms. The item whether bacterial populations in the substrates altered by gut-associated bacteria of earthworms or the differences were only quantitative is still unclear.

4.2 Proteolytic activity of the soil and earthworm-modified microbial communities

According to the inhibitor assay, metalloproteases were the main enzymes caused unspecific proteolytic activity of the soil aqueous extract. Proteases inhibited with aprotinin and leupeptine (trypsin, chymotrypsin, trypsin-like and some other proteases) were not abundant and less active. Unspecific proteolytic activity was significantly higher in water-soluble content from the earthworm casts than in soil extracts and was mainly comprised by proteases inhibited with

aprotinin and leupeptin. At the same time enzymatic activity of metalloproteases in the soil and earthworm casts was on approximately same level.

Metallopeptidases are present in both prokaryotes and eukaryotes and often have much in common, in terms of both, structure and biochemical properties (Rawlings and Barrett, 1995). Eukaryotes (as well as bacteria) can produce proteases of trypsin and chymotrypsin family. Chymotrypsin is mainly produced by animals, trypsin-like enzymes are found in actinomycetes of the genera *Streptomyces* and *Saccharopolyspora*, and in the fungus *Fusarium oxysporum* (Rawlings and Barrett, 1994). The enhanced unspecific proteolytic activity of water-soluble earthworm cast content was clearly based on trypsin- and chymtrypsin-like proteases derived from earthworm gut system (earthworm gut tissue and host gut-associated organisms).

Proteolytic activity of the earthworm lumen content was previously reported for certain tropical earthworms (Mishra *et al*., 1980) and for *L. terrestris* (Tillinghast *et al*., 2001). Protease activity in the cast of earthworm *Eudrilus eugeniae* was lower than in the pig slurry (Aira *et al*., 2005). Several alkaline serine proteases were purified from tissues of earthworms *L. rubellus* and *E. fetida* and later characterized. Six alkaline proteases from *L. rubellus* consisted of single polypeptide chain encoded by different genes. They hydrolyzed various proteins and the caseinolytic and fibrinolytic activities of the enzymes was much higher than plasmin. Those proteases showed similarity to mammalian serine proteases, however, neither arginine nor lysine residues were present in the autolysis region. The enzymes were clearly distinct from other fibrinolytic enzymes, such as plasmin and urokinase in amino acid composition. The proteases had long-term stable activity at room temperature and a wide range of pH, being strongly resistant to organic solvents and detergents. They were suggested to be trypsin-like and chymotrypsin-like serine proteases (Mihara *et al*., 1991; Nakajima *et al.,* 1993, 2002, 2003; Sugimoto and Nakajima, 2001; Cho *et al*., 2004; Hu *et al*., 2005). Most of isozymes were strongly or partially inhibited with aprotinine (Nakajima *et al.,* 2003) but some recombinant was sensitive to EDTA (Hu *et al*., 2005). Seven purified proteins from *E. fetida* were characterized as a “living fossil” in the evolution track of the chymotrypsin protein family (Wang *et al*., 2003, 2005). Enzymes purified from *Lumbricus rubellus* and *E. fetida* exhibited high homology each to other (Wang *et al*., 2003; Zhao et al., 2003). Yang and colleagues (1997) characterized SDS-activated fibrinolytic enzyme from *Eisenia fetida* to consist of two subunits multicatalytic enzyme, which has more than one active center. Leupeptin and aprotinin did not affect this enzyme and the protease inhibitor E-64 even activated it.

Thus, even a single species of the earthworm does produce a wide range of different proteases. Some of them are similar to those from other species, but certain enzymes are probably unique. Tang and colleagues (2002) suggested that this enzymatic diversity is very advantageous for

earthworms to digest proteins efficiently under specific living and nutritional conditions during migration through the substratum or upon passage different nutritional substrate through their gut. However, the source of the proteases we detected in our study is rather uncertain. The enzymes could be released from destructed bacteria (Gibson *et al.,* 1989) and could also be produced in the tissues of earthworm gut (Tillinghast *et al*., 2001) and transported into blood through the intestinal wall (Fan *et al*., 2001). That looks reasonable because physiologically active part of soil bacterial community did not significantly increase its number upon passage (ratio EUB338/DAPI) and proteolytic activity of proteases inhibited with EDTA (most probably bacterial metalloproteases) in the earthworm cast samples was the same or almost the same as in the soil aqueous extracts. Therefore we have concluded that proteases enriched in the earthworm gut environment and thus caused the high unspecific proteolytic activity of water-soluble content of the cast were derived from earthworm tissues.

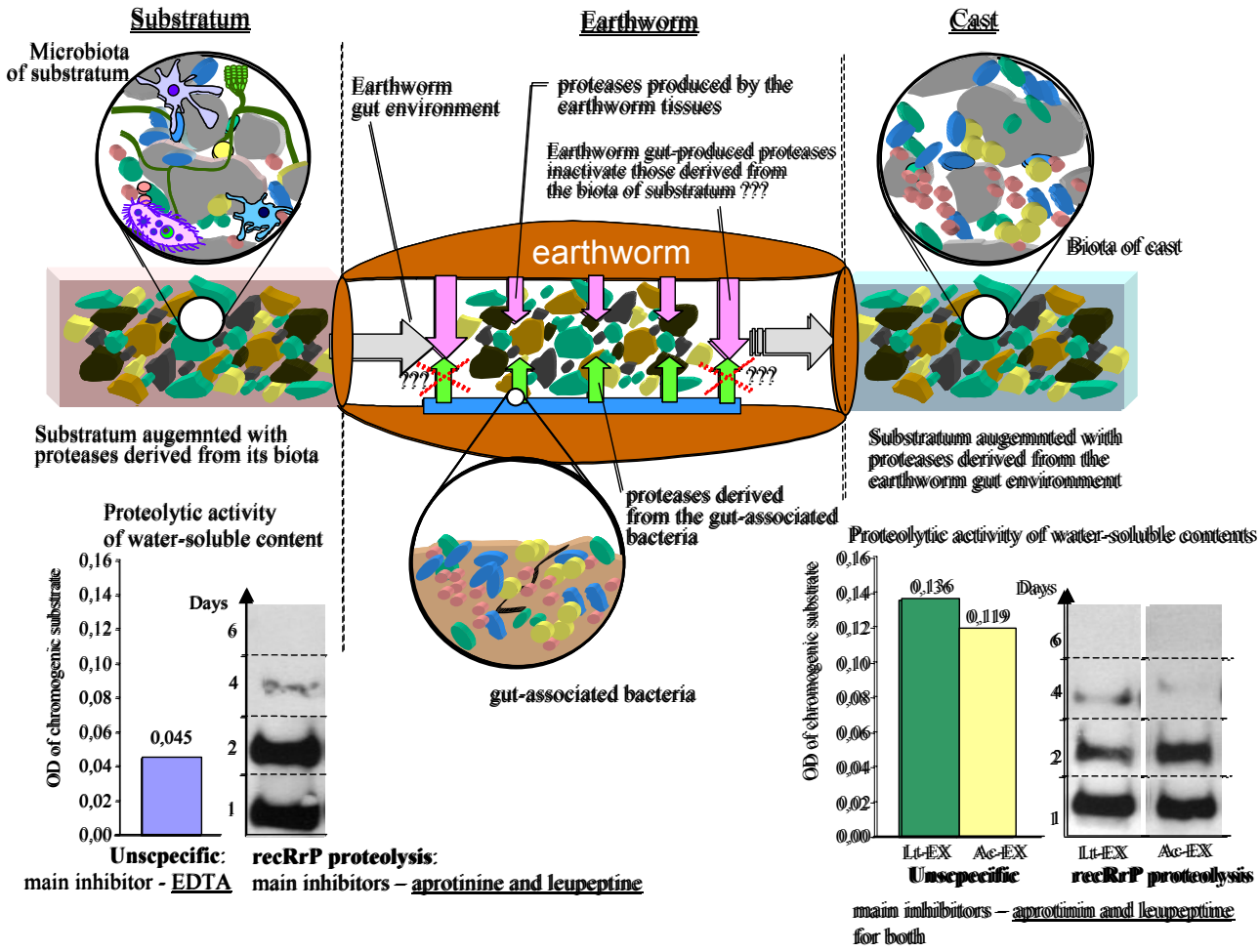


Figure 32. The scheme of protease-producing agents in variuos compartments of earthworm-associated environment. Substratum proteases (mostly inhibited with EDTA) get augmented with enzymes derived from the earthworm gut environment (mostly inhibited with aprotinin and leupeptine), while recPrP proteolysis kept the same rate. Lt-EX – *L. terrestris* and Ac-EX – *A. caliginosa* casts.

In contrast to unspecific proteolytic activity of the aqueous extracts, recombinant prion proteolysis activity in all samples was comparable among the variants. Degradation of recPrP was observed only in the aqueous extracts without inhibitors and with added Inhibitor Cocktail Set II uninhibited trypsin- and chymotrypsin-family proteases. DMSO used as a solvent and protease inhibitor E-64 also presented in the Inhibitor Cocktail Set II could activate enzymes (Yang et al., 1997) and caused a tiny increase of recPrP proteolysis observed in the variant with the Set II in comparison with variant without inhibitors. Protease Inhibitor Cocktail Set III contained aprotinin and leupeptine blocked completely recPrP-degrading enzymatic activity. As we have concluded above, the earthworm gut produced the proteases that greatly increased unspecific proteolytic activity of aqueous extract from the casts. But these proteases did not remarkably affect the recPrP degradation in water-soluble content from the casts. Thus, we think that proteases derived from earthworm gut system (neither organisms associated with earthworm intestine nor gut tissue) do not digest the prion, and recPrP degradation was performed primarily by the proteases derived from soil microorganisms. Those proteases belong to trypsin- and chymotrypsin-family produced by bacteria and eukaryotes, as it was mentioned above and showed low unspecific proteolytic activity.

Pure microbial cultures exhibited different capacities for prion proteolysis but in the most cases recPrP-degrading microbes reduced recPrP amounts to the levels below the detection. Taxonomic distribution of the bacterial isolates capable for recPrP proteolysis was diverse but only 19% of isolates in total showed capacity for recombinant prion proteolysis (Fig. 33). *Alpha*- and *Betaproteobacteria* have shown low ability for recPrP digestion (1% from each class among the total estimated isolates). *Gammaproteobacteria* (mostly genus *Pseudomonas*), *Actinobacteria*, and *Bacilli* (only genus *Bacillus*) were more capable: 7%, 6%, and 4% respectively (Fig. 33). Bacteria of *CFB* group were not abundant among the isolates (even being numerous in the communities) and none of isolates showed recPrP proteolysis activity.

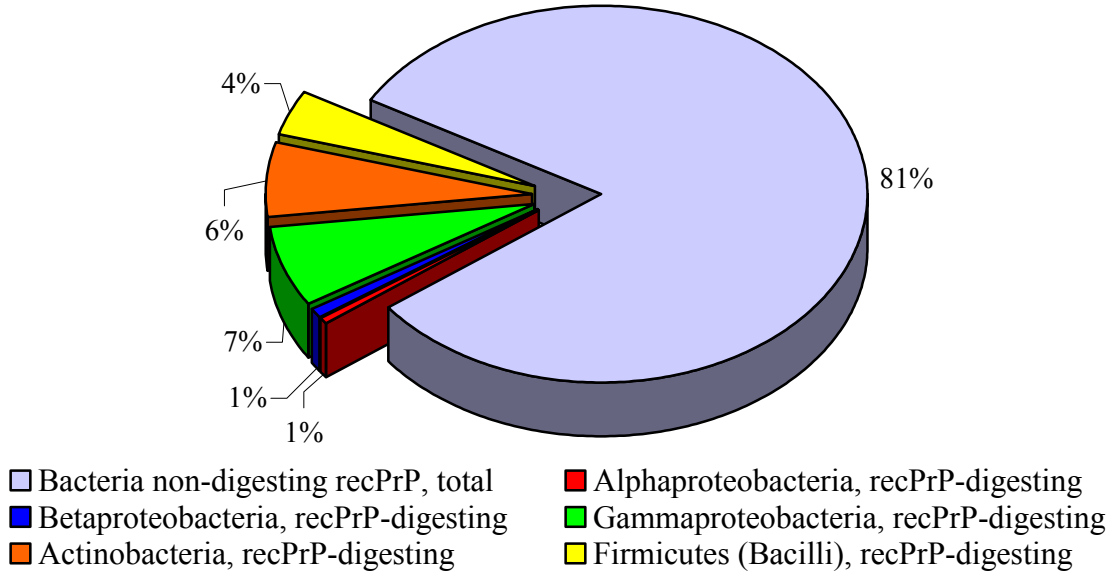


Figure 33. Correlation between digesting and not digesting recPrP bacterial isolates.

Most of bacteria able to digest recPrP have hydrolyzed whole molecule, at least both epitope sites of it were destroyed. But some species, for instance *Pantoea agglomerans 571-2* clearly degraded only N-terminus of the recombinant prion (epitope of PrPc248), while *Aeromonas sobria 368-2* and *Pseudomonas sp. 9* have digested epitope of antibody VRQ14 (α-helix and interhelix loop). Fungi were very active in recPrP digestion. Six among eight the most common isolates appeared recPrP-proteolysis activity, digesting whole recPrP molecule.

Low proteolytic activity towards recPrP of aqueous extracts from studied soil and earthworm cast could be explained with low quantity of bacterial groups potentially able for prion proteolysis (*Gammaproteobacteria*, *Bacilli* (*Firmicutes*) and *Actinobacteria*). The population densities of all these groups went down upon the transit through the gut and their total number in bacterial population did not exceed 11%. Fungi are also efficient in recPrP digestion but they also were eliminated from community upon gut passage (Kristufek *et al.,* 1992; Toyota and Kimura, 1994; Schönholzer *et. al.,* 2002).

5. Conclusions

- Bacteria in the substrate undergo severe changes upon the gut passage. Independently of the earthworm species and initial substrata, the common rules are: (i) increase of relative densities of *Cytophaga-Flavobacteria* group of *Bacteroidetes* during the transit; (ii) densities of *Firmicutes* and *Gammaproteobacteria* decreased in the earthworm cast compared to substrata; (iii) numbers of *Alpha*-, *Betaproteobacteria*, and *Actinobacteria* varied.

- Many bacterial isolates (depending on taxonomic affiliation, up to 33%) from earthworms did digest recPrP in pure cultures. *Gammaproteobacteria*, *Bacilli*, *Actinobacteria,* and fungi were the most active potential degraders of recPrP *in vitro*.

- Recombinant recPrP was definitely degraded *in vitro* in aqueous extracts of the earthworm casts and the soil. This process took under given conditions up to 2-6 days. However, additional experiments with labeled recPrP should be performed in the future to unambiguously confirm the same in the system with presence solid soil particles and organic matter.

- Non-specific proteolytic activity of soil strongly increased during the transit through the earthworm gut. The major contribution to that were the earthworm-produced enzymes. However, this augmentation along with modification of microbial population in the earthworm gut environment did not enhance the recPrP digestion. Most likely, the active enzymatic fraction for recPrP proteolysis in the soil enzyme pool was constituted by trypsin- and chymotrypsin-like proteases. The contribution to this pool of the earthworm itself and earthworm gut microflora seems to be minimal. Thus, studied microbial-earthworm gut systems do not produce proteases notably affecting the prion proteolysis.

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Supplementary materials

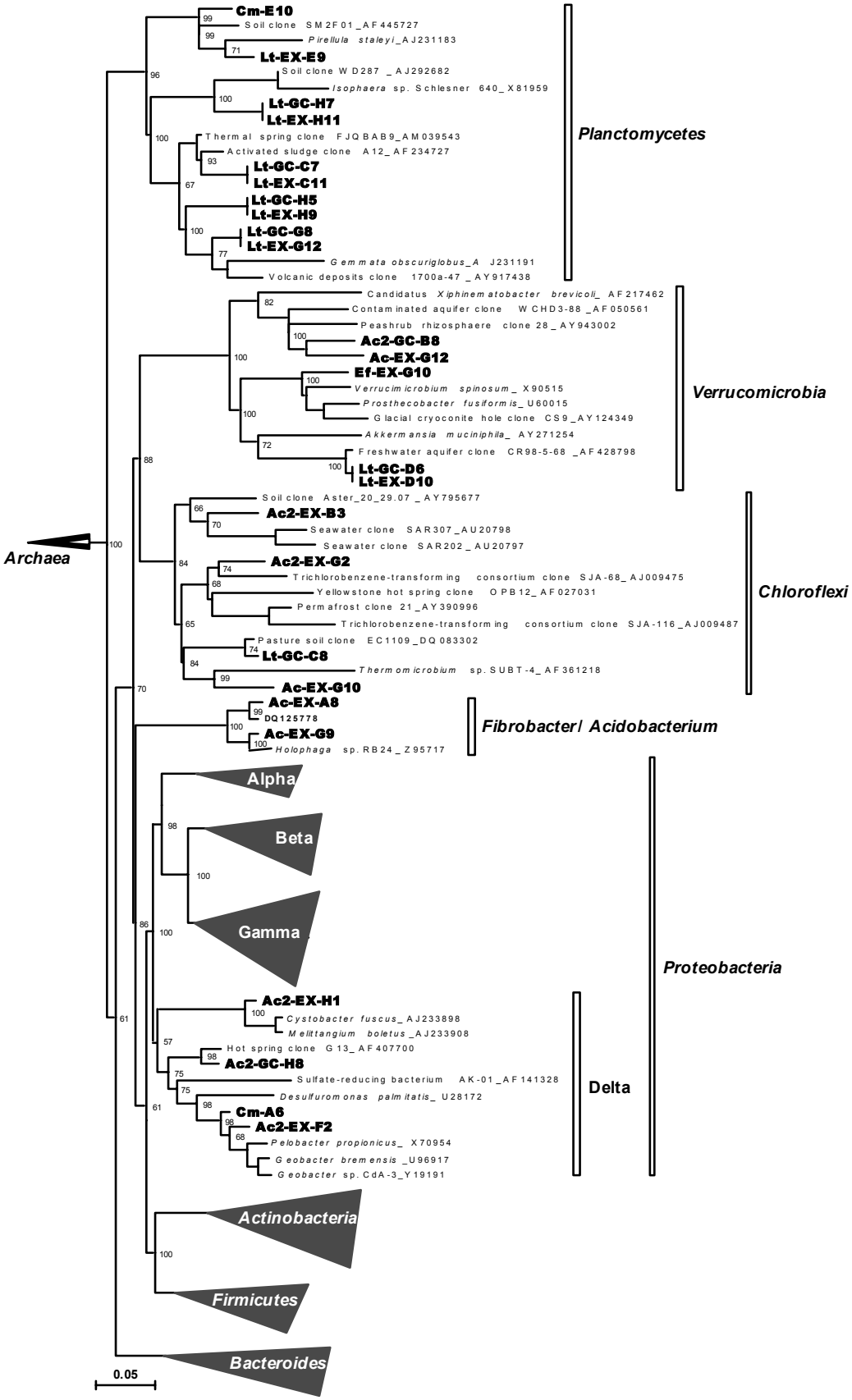

Figure 34. Overview of bacterial diversity in the soil, compost, gut content and casts of three earthworm species: SPs1 (autumn-collected soddy-podzolic soil); SPs 2 (spring-collected soddy-podzolic soil); Cm – compost; Lt – *L. terrestris*; Ac – autumn-collected *A. caliginosa*; Ac2 – spring-collected *A. caliginosa*; Ef – *E. fetida*; GW – gut wall; GC – gut content; EX – excrements.

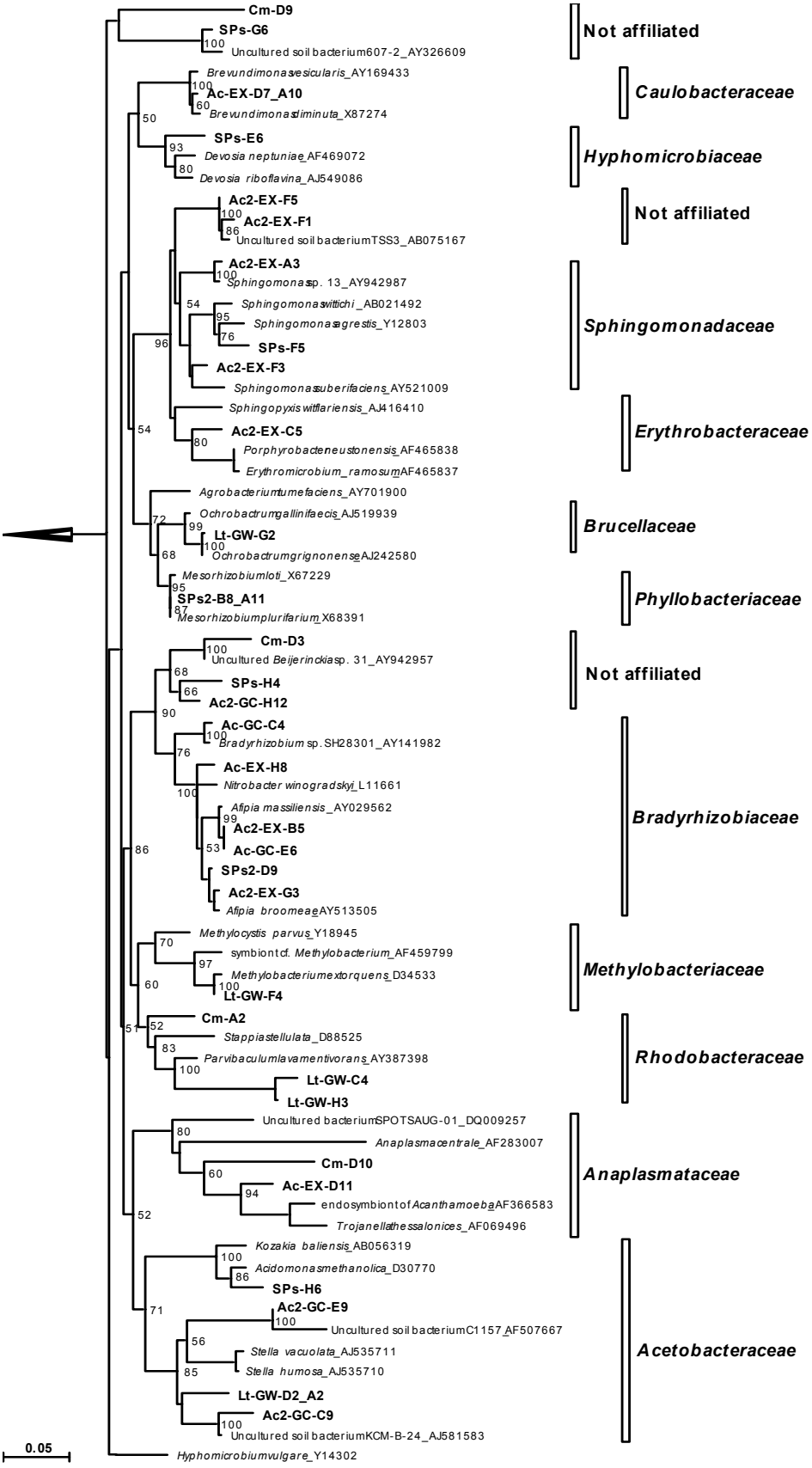

Figure 35. Phylogenetic affiliation of the clones from *Alphaproteobacteria* in the soil, compost, gut content and casts of three earthworm species: SPs1 (autumn-collected soddy-podzolic soil); SPs 2 (spring-collected soddy-podzolic soil); Cm – compost; Lt – *L. terrestris*; Ac – autumn-collected *A. caliginosa*; Ac2 – spring-collected *A. caliginosa*; Ef – *E. fetida*; GW – gut wall; GC – gut content; EX – excrements.

Figure 36. Phylogenetic affiliation of the clones from *Betaproteobacteria* in the soil, compost, gut content and casts of three earthworm species: SPs1 (autumn-collected soddy-podzolic soil); SPs 2 (spring-collected soddy-podzolic soil); Cm – compost; Lt – *L. terrestris*; Ac – autumn-collected *A. caliginosa*; Ac2 – spring-collected *A. caliginosa*; Ef – *E. fetida*; GW – gut wall; GC – gut content; EX – excrements.

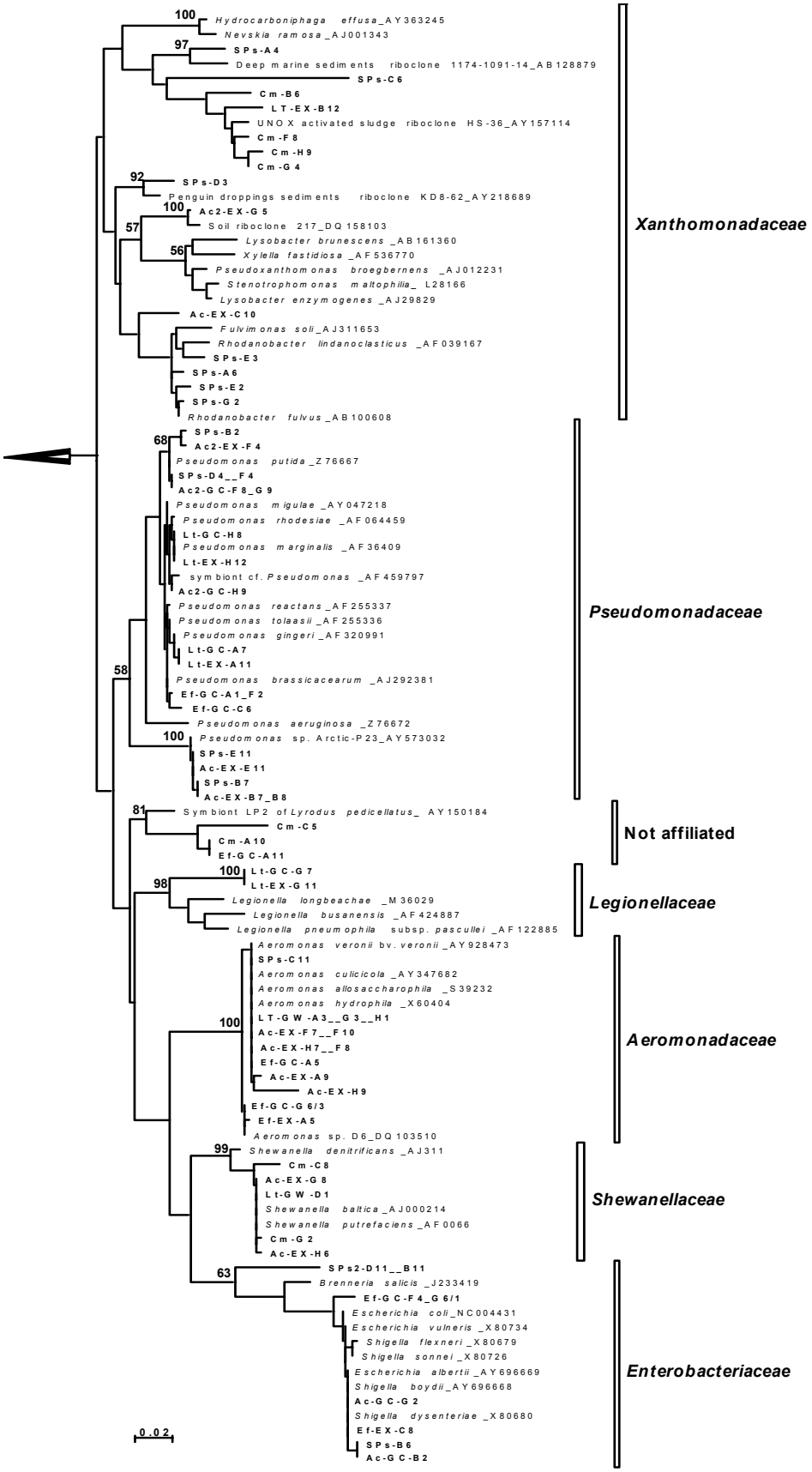

Figure 37. Phylogenetic affiliation of the clones from *Gammaproteobacteria* in the soil, compost, gut content and casts of three earthworm species: SPs1 (autumn-collected soddy-podzolic soil); SPs 2 (spring-collected soddy-podzolic soil); Cm – compost; Lt – *L. terrestris*; Ac – autumn-collected *A. caliginosa*; Ac2 – spring-collected *A. caliginosa*; Ef – *E. fetida*; GW – gut wall; GC – gut content; EX – excrements.

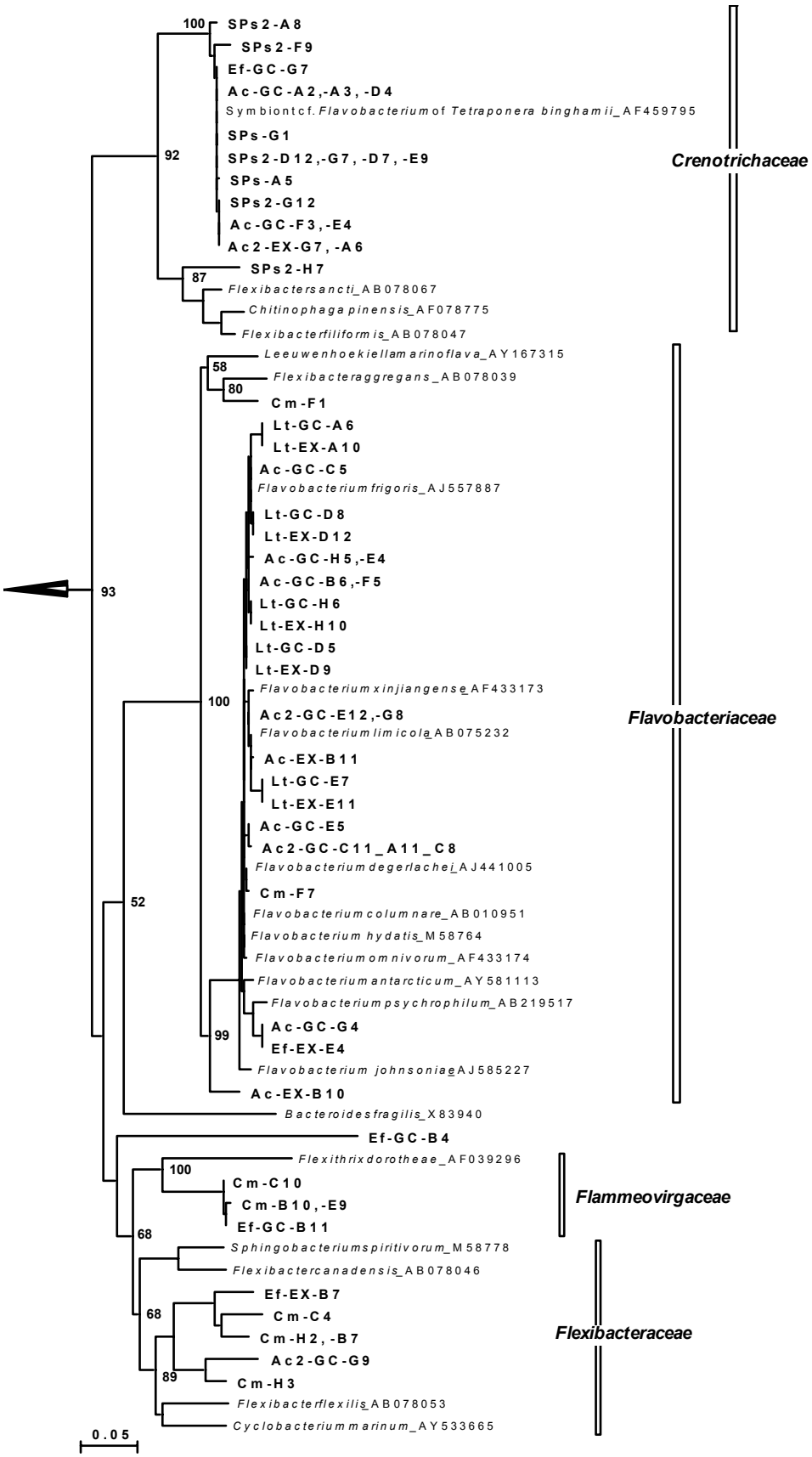

Figure 38. Phylogenetic affiliation of the clones from *CFB* in the soil, compost, gut content and casts of three earthworm species: SPs1 (autumn-collected soddy-podzolic soil); SPs 2 (spring-collected soddy-podzolic soil); Cm – compost; Lt – *L. terrestris*; Ac – autumn-collected *A. caliginosa*; Ac2 – spring-collected *A. caliginosa*; Ef – *E. fetida*; GW – gut wall; GC – gut content; EX – excrements.

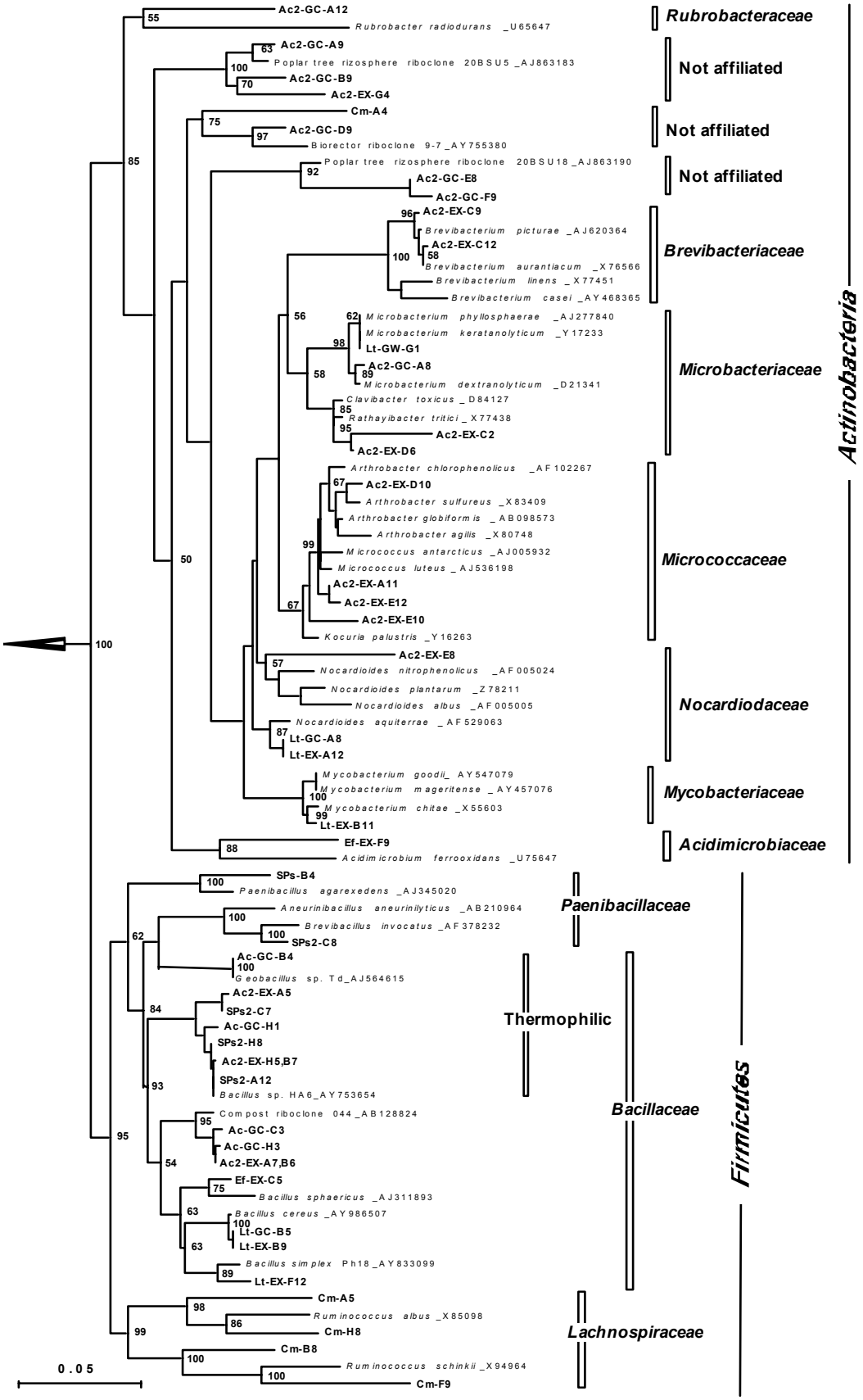

Figure 39. Phylogenetic affiliation of the clones from *Actinobacteria* and *Firmicutes* in the soil, compost, gut content and casts of three earthworm species: SPs1 (autumn-collected soddy-podzolic soil); SPs 2 (spring-collected soddy-podzolic soil); Cm – compost; Lt – *L. terrestris*; Ac – autumn-collected *A. caliginosa*; Ac2 – spring-collected *A. caliginosa*; Ef – *E. fetida*; GW – gut wall; GC – gut content; EX – excrement.

Curriculum Vitae

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Publications

Nechitaylo, T.J., & Peskova T.J. Effect of low concentrations of heavy metals on embryonal and larval development of amphibia. (Poster) 11. Interrepublical Scientific Conference "Live Issues of Ecology and Natural Protection of South Regions of Russia and Frontier Territories", Krasnodar (1999).

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